REMARKS

Claims 576-825 are pending and continue to be presented for prosecution in this application. No claim changes have been made by this paper.

This paper supplements Applicants' May 28, 2002 Reply Under 37 C.F.R. §1.111 with respect to two of the rejections set forth in the November 26, 2001 Office Action. In their May 28, 2002 Reply, Applicants addressed the first rejection under 35 U.S.C. §102 (anticipation)[1] and the seventh rejection under 35 U.S.C. §103 (obviousness)[2] on pages 67 and 83, respectively. The information contained in this paper in the form of a declaration and exhibits will serve to clarify Applicants' position further on both issues.

The First Rejection Under 35 U.S.C. §102

The text of Applicants' response to the first anticipation rejection is repeated below:

> In response, Applicants respectfully point out that the Ward '955 Patent limits the attachment of the non-radioactive labels to the non-disruptive base positions of the pyrimidine, purine or deazapurine, namely, the 5-, 8- and 7-positions, respectively. In contrast, the claims in the Engelhardt application are directed to compositions in which the non-polypeptide, non-radioactive label moiety Sig is attached to the phosphate moiety -- not even to the base, let alone to the aforementioned Ward positions (the 5-, 8- or 7-positions of a pyrimidine, purine or

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[1] In the §102 rejection, claims 454-461, 463-474, 476-489, 491-502, 504-518, 520-531, 533-546, 548-559 and 561-567 were rejected for anticipation by Ward et al., U.S. Patent No. 4,711,955. The text of the rejection is set forth on pages 4-5 in the November 26, 2001 Office Action.

[2] In the §103 rejection, 462, 490, 519, and 547 were rejected as being obvious over the Ward '955 Patent, as applied to claims 454-461, 463-474, 476-489, 491-502, 504-518, 520-531, 533-546, 548-559 and 561-567 above, and further in view of Falkow et al., U.S. Patent No. 4,358,535. The text of the rejection is set forth on pages 18-19 in the November 26, 2001 Office Action.

> deazapurine, respectively). There are at least two significant reasons why it is incorrect and improper to characterize the specific base labeling positions in the Ward '955 Patent for attaching non-radioactive labels as being indirectly attached to the phosphate moiety through the linkage of the sugar.
>
> First, the sugar is a distinct element of a nucleotide and it is not recognized in the art to my knowledge as being an indirect linkage of the phosphate moiety to the base moiety. A person of ordinary skill in the art would simply not consider the attachment to the base moiety in a nucleotide to be an indirect linkage to the phosphate moiety in a nucleotide.
>
> Second, it should not be overlooked that the three elements making up a nucleotide (sugar, phosphate and base) are not only different structurally, but they are different chemically, such that these elements are subject to *different* chemical reactions. Again, a person of ordinary skill in the art would simply not treat the sugar, phosphate and base moieties in a nucleotide as interchangeable elements.
>
> [May 28, 2002 Reply, page 67]

In supplementing the remarks above, Applicants are pleased to submit the Declaration of Dr. Alexander A. Waldrop, III, a copy of which is attached as Exhibit A. Dr. Waldrop is a scientist and inventor with more than twenty-five years of research experience who is one of the inventors named on the cited Ward '955 Patent. His credentials are listed on his *curriculum vitae* attached to his Declaration as Exhibit 1. His research experience, education and training are set forth in Sections 1-4 (pages 1-4) in his Declaration (Exhibit A). As indicated in Section 9 (page 14), Dr. Waldrop possesses the level of skill and knowledge of a person of at least ordinary skill in the art to which the present application and invention pertains.

After providing some background to his work as one of the inventors of the cited Ward '955 Patent (Sections 11A through 11C, pages 16-19), Dr. Waldrop addresses the first anticipation rejection in Section 12 in his Declaration, beginning on page 19, and continuing through the first two paragraphs on page 25.

Applicants respectfully request that consideration be given to Dr. Waldrop's remarks, including Exhibits 2-12 which are attached to his Declaration (Exhibit A).

In light of Dr. Waldrop's Declaraton and their earlier response, Applicants respectfully request that the first anticipation rejection be withdrawn.

The Seventh Rejection Under 35 U.S.C. §103

The text of Applicants' response to the seventh obviousness rejection is repeated below:

> It is respectfully submitted that it would not have been obvious to one of ordinary skill in the art to have modified Ward's compound to include a glycosidic linkage instead of peroxidase, in view of Falkow's '535 Patent and its disclosure of enzymes as labels. One would simply not have arrived at the Engelhardt invention from a combined reading of the Ward and Falkow patents. Ward's Patent does not disclose among its compounds a non-polypeptide, non-radioactive label moiety Sig attached to the nucleotidyl phosphate moiety in an oligo- or polynucleotide, as generally set forth in the Engelhardt claims. Furthermore, other Engelhardt claims recite a non-polypeptide, non-radioactive label moiety Sig, or the members of Sig (biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, a hapten or a combination of any of the foregoing), or that Sig is covalently attached to the phosphate moiety through a chemical linkage comprising a polypeptide or a protein. The element Sig in the Engelhardt claims is not an enzyme as disclosed in the Falkow '535 Patent. Thus, the addition of the Falkow '535 Patent does not provide the necessary disclosure which would have motivated or allowed a person of ordinary skill in the art to arrive at the claims in the Engelhardt application from a combined reading of both the Ward and Falkow patents. [May 28, 2002 Reply, page 83]

In further support of the patentability of their claimed invention, Applicants are pleased to refer to Dr. Alexander A. Waldrop, III's Declaration (Exhibit A). His

remarks on the issue of the seventh obviousness rejection are set forth on page 25, last paragraph, continuing through page 26, first two paragraphs. It is respectfully requested that consideration be given to Dr. Waldrop's remarks, and that the rejection under §103 be withdrawn.

Favorable action is respectfully solicited.

* * * * * * *

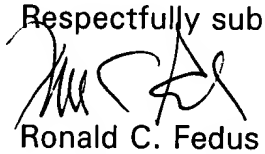
SUMMARY AND CONCLUSIONS

Claims 576-825 continue to be presented for examination in this application. No claims have been amended, added or canceled by this paper.

No fee or fees are believed due for this paper, a Request For Extension Of Time (3 months) and authorization for the fee therefor having been submitted earlier with Applicants' May 28, 2002 Reply Under 37 C.F.R. §1.111. In the event that any other fee or fees are due, however, authorization is hereby given to charge the amount of any such fee(s) to Deposit Account No. 05-1135, or to credit any overpayment thereto.

If a telephone conversation would further the prosecution of the present application, Applicants' undersigned attorney request that he be contacted at the number provided below.

Respectfully submitted,



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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Engelhardt, *et al.*

Serial No.: 08/479,997

Filed: June 7, 1995

Group Art Unit: 1656

Ex'r: Alexander H. Spiegler

For: **OLIGO- OR POLYNUCLEOTIDES COMPRISING PHOSPHATE MOIETY LABELED NUCLEOTIDES**
(As Previously Amended)

South Portland, Maine 04106

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF DR. ALEXANDER A. WALDROP, III

I, Alexander A. Waldrop, III, hereby declare as follows:

1. Since 2000, I have been organizing a new company located at the Center for Environmental Enterprise (CEE), South Portland, Maine.[1] In my present work at CEE, I have further characterized 9-acridinecarbonylimidazole (AcriGlow™ 301) and its reactions with peroxide in various buffers and solvents. This technology can be used to develop chemiluminescent assays for a wide variety of substances, including assays for use in medical diagnostics and in the environmental field.

[1] **CEE is a private, non-profit corporation funded by the State of Maine as a business incubator. Affiliated with Southern Maine Technical College, CEE helps new and young firms like my own to commercialize technologies in the environmental field.**

My focus is in these two areas. I have served as a consultant for a number of companies, including Brims Ness, Maine Standards and Enzo Biochem, Inc. Prior to my present position, I was Visiting Scientist at the Maine Medical Center Research Institute, South Portland, Maine, from 1994 to 2000. While at MMCRI, I synthesized and characterized a modified acridancarboxylic acid ester and I demonstrated its substrate activity with horseradish peroxidase (HRPO). I also synthesized and characterized activated 9-acridinecarboxylic acid derivatives. Further, I developed high sensitivity chemiluminescent assays for glucose oxidase and alkaline phosphatase using these derivatives. I also conducted HPLC of acridine derivatives and synthetic oligonucleotides. From 1992 to 1993, I was Research Scientist at IDEXX Laboratories, Inc., Westbrook, Maine. At IDEXX, I worked on optimizing HRPO assay systems. Earlier, from 1985 to 1992, I was Staff Scientist at Gen-Probe, Inc., San Diego, California. At Gen-Probe, I synthesized and helped to design acridinium esters. I contributed to methods for stabilizing acridinium esters. This is the subject matter of U.S. Patent No. 4,950,613, on which I am named as a co-inventor.[2] I contributed to methods for improving the elution of nucleic acids from solid supports. This is the subject matter of U.S. Patent No. 5,599,667, on which I am also named as a co-inventor.[3] In that line of research, I characterized acridinium esters by HPLC, UV and chemiluminescence. My professional experience is listed and described in my *curriculum vitae* attached to this Declaration as Exhibit 1.

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[2] The citation is Arnold, Lyle J., Waldrop, Alex A., III, Hammond, Philip W., "Protected Chemiluminescent Labels," U.S. Patent No. 4,950,613, issued on August 21, 1990.
[3] The citation is Arnold, Lyle, J., Nelson, Norman C., Reynolds, Mark A., Waldrop, Alex A., III; "Polycationic Supports and Nucleic Acid Purification, Separation and Hybridization," U.S. Patent No. 5,599,667, issued on February 4, 1997.

2. In terms of my education, including my research training and experience, I received my bachelor of science degree (B.S.) in 1970 from the University of Virginia, graduating with high distinction (*magna cum laude*). Later I received my doctoral degree (Ph.D.) in biophysics in 1977 from The Johns Hopkins University, Baltimore, Maryland. There, in the Department of Biophysics, I trained as a predoctoral fellow from 1970-1977 in the laboratory of Dr. Michael Beer. In my graduate work, I developed multiple heavy atom stains for electron microscopy of nucleic acids. The title of my doctoral dissertation was "Chemical Studies of *bis*(Pyridine)osmate(VI) Esters and the Mercury Enhancement of Osmium Labelling of Polynucleotides" [Disssertation Abstracts International 38(11-B):5354+ (194 pp.) (1978)]. As a postdoctoral fellow, I worked in the laboratory of Dr. David C. Ward at Yale University, New Haven, Connecticut from 1977-1980. While at Yale I used reactions with heavy metal intermediates to synthesize modified pyrimidine nucleotides and I helped to demonstrate their incorporation, *in vitro*, into nucleic acids. This led to the development of novel nucleotide analogs which are used for *in situ* gene detection, as disclosed and claimed in several U.S. patents (Nos. 4,711,955; 5,328,824; 5,449,767; and 5,476,928) on which I am named as a co-inventor.[4] These nucleotide analogs include biotinylated nucleotides which have been selling commercially for years.

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[4] All four of these U.S. patents name David C. Ward, Pennina R. Langer and Alexander A. Waldrop, III, as co-inventors. U.S. Patent No. 4,711,955 is titled "Modified Nucleotides and Methods of Preparing and Using Same" and it isssued on December 8, 1987. U.S. Patent No. 5,328,824 is titled "Methods of Using Labeled Nucleotides" and it issued on July 12, 1994. U.S. Patent No. 5,449,767 is titled "Modified Polynucleotides and Methods of Preparing Same," having issued on September 12, 1995. The fourth, U.S. Patent No. 5,476,928, is titled "Modified Nucleotides and Polynucleotides and Complexes Formed Therefrom," and it issued on December 19, 1995.

3. After my postdoctoral work, I was Assistant Professor of Chemistry at the University of Virginia, Charlottesville, Virginia, from 1980-1982. While working in the Department of Chemistry, I prepared nucleotide derivatives of tubercidin and characterized the allylamine derivatives. I taught biophysical chemistry to undergraduate students. From 1982-1985, I was Research Associate in the Department of Microbiology, University of Virginia. At UVA, I developed a new DNA sequencing method similar to Dr. Fred Sanger's approach, but one in which the method leaves functional 3' ends which can be extended under conditions forcing misincorporation and then are ligated to produce a set of point mutants. Alternatively, the method can be carried out without misincorporation to generate a set of deletion mutants. I also synthesized a series of 5'-thymidine triphosphate derivatives containing a 3'-phosphate mono-, di-, or triester group. I showed that these analogs were not substrates for T4 or Klenow DNA polymerase. I also developed a simple, rapid gel filtration method for purifying and desalting nucleotides. In addition, I synthesized an analog of dUTP containing an EDTA group and I showed that it can be enzymatically incorporated into DNA. My education and research experience are set forth in my CV (Exhibit 1).

4. I am the author of five scientific publications and I am named as an inventor on seven U.S. patents. My publications and patents are listed in my CV (Exhibit 1).

5. I have been asked by Enzo Life Sciences, Inc. to review, as its scientific consultant, significant portions of the prosecution history of United States Patent Application Serial No. 08/479,997, filed on June 7, 1995 in the name of Dean L. Engelhardt, *et al.* as inventors. The title of the Engelhardt application is "Oligo- or Polynucleotides Comprising Phosphate Moiety Labeled Nucleotides." Included for my review were the following documents: the patent specification filed on June 7,

1995 (but that I have been informed claims priority back to June 23, 1982); the previously pending claims (454-567) in this application; the November 26, 2001 Office Action; the December 27, 2001 Interview Summary, and various prior art documents cited in the aforementioned office action. The cited documents that I have reviewed include two scientific papers by Mark J. Halloran and Charles W. Parker ["The Preparation of Nucleotide-Protein Conjugates: Carbodiimides As Coupling Agents," Journal of Immunology 96:373-378 (1966); "The Production of Antibodies to Mononucleotides, Oligonucleotides and DNA," Journal of Immunology 96:379-385)], and two U.S. patents [Ward et al., U.S. Patent No. 4,711,955; and Falkow et al., U.S. Patent No. 4,358,535]. I am also the same Alexander A. Waldrop, III named on the aforementioned Ward patent cited in the November 26, 2001 Office Action. I have also reviewed two declarations that were submitted in the Engelhardt application: Declaration of Dr. Cheryl H. Agris, Attorney At Law (In Support of the Written Description, Enablement & Non-Obviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997); and Declaration of Dr. Dean L. Engelhardt In Support of Adequate Description and Enablement. I have also been informed that the Agris Declaration and the Engelhardt Declaration were originally submitted to the U.S. Patent Office on January 18, 2001 and November 27, 1997, respectively. As part of my review, I read a Reply Under 37 C.F.R. §1.111 that was filed on May 28, 2002 in response to the November 26, 2001 Office Action. Included in the May 28, 2002 Reply was a new set of claims 576-825 which I also reviewed.

6. Based upon my review of the claims (576-825) that were submitted to the U.S. Patent Office in the May 28, 2002 Reply, I believe that the invention in the Engelhardt application is directed to oligo- or polynucleotides comprising phosphate moiety labeled nucleotides. Such claimed oligo- or polynucleotides are useful as

hybridization probes for detecting nucleic acids of interest. More particularly, I believe that a third of the claimed embodiments in the Engelhardt application are directed to *non-polypeptide*, non-radioactive label moieties attached to the phosphate moiety of a modified nucleotide in an oligo- or polynucleotide (claims 576-657). Another third of Engelhardt's claimed embodiments (claims 658-735) are directed to recited members for the non-radioactive label moiety Sig in such modified nucleotides in an oligo- or polynucleotide. Such members can take the form of biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, a hapten and combinations of any of the foregoing. None of the following are included in the preceding list of Sig members: a polypeptide, a protein and an enzyme. The remaining third of Engelhardt's claimed embodiments (736-813) are directed to an oligo- or polynucleotide comprising at least one modified nucleotide in which a non-radioactive moiety label Sig is directly detected when indirectly attached to the phosphate moiety through a chemical linkage comprising a polypeptide or a protein. Finally, several dependent claims (814-825) define embodiments wherein Sig is covalently attached to PM or the phosphate through a chemical linkage comprising a polypeptide or a protein (claims 814, 817, 820 and 823). Other dependent claims define such polypeptide as comprising polylysine (claims 815, 818, 821 and 824) or such polypeptide or protein as being selected from avidin, streptavidin and anti-hapten immunoglobulin (claims 816, 819, 822 and 825).

A. Based upon my review, I believe that claims 576-595 describe one of the major compositions in the Engelhardt application. As set forth in claim 576, the claimed oligo- or polydeoxyribonucleotide, which is complementary to a nucleic acid of interest or a portion thereof, comprises at least one modified nucleotide having the formula

Sig—PM—SM—BASE

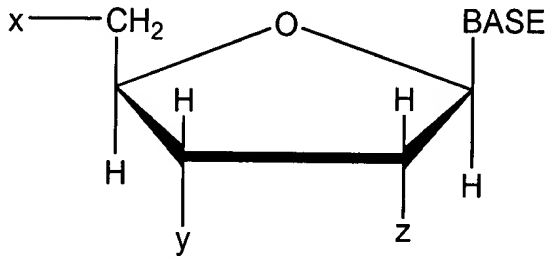
wherein PM is a phosphate moiety attached to SM, a sugar moiety, and BASE is a base moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, the BASE being attached to SM. Sig is covalently attached to PM directly or through a chemical linkage, and Sig comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when the modified nucleotide is incorporated into the oligo- or polydeoxyribonucleotide or when the oligo- or polydeoxynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof. I believe that claims 577-595 depend from claim 576 and are directed to various other specific embodiments, such as the nature of Sig (claims 577-578 and 586-591); the covalent attachment of Sig (claims 579 and 592); the chemical linkage (claims 580-584); the nature of PM (claim 585); the nature of SM (claim 593-594); and the inclusion of at least one ribonucleotide (claim 595).

(i) I believe that claims 658-676 differ from claims 576-595 in two respects. First, independent claim 658 does not recite the term "non-polypeptide" for the non-radioactive label moiety Sig. Second, claim 658 recites specific members for Sig (biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, a hapten and a combination). The claims that depend from claim 658 are also directed to specific embodiments, such as nature of Sig (claims 659-660 and 668-672); the covalent attachment of Sig (claims 661 and 673); the chemical linkage (claims 662-666); the nature of PM (claims 667); the nature of SM (claims 674-675); and the inclusion of at least one ribonucleotide (claim 676).

(ii) I also believe that claims 736-754 differ from the above-described claims 576-595 and 658-676 as follows. Claim 736 is independent and it recites that Sig is covalently attached to PM through a chemical linkage comprising a polypeptide or a protein. Thus, for the non-radioactive label moiety Sig, claim 736 recites neither the term "non-polypeptide" nor the Sig members. Claims 737-754 are directed to further more specific embodiments of claim 736. These dependent claims define the nature of Sig (claims 737-738 and 742-750); the covalent attachment of Sig (claims 739 and 751); the chemical linkage (claims 740 and 748-750); the nature of PM (claim 741); the nature of SM (claims 752-753); and the inclusion of at least one ribonucleotide (claim 754).

B. From my review I believe that claims 596-616 define another aspect of the Engelhardt invention. As set forth in claim 596, the invention claimed in the Engelhardt Declaration is also directed to an oligo- or polydeoxyribonucleotide which is complementary to a nucleic acid of

interest or a portion thereof. Such oligo- or polydeoxyribonucleotide comprises at least one modified nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, the BASE being attached to the 1′ position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine. The elements x, y and z are each selected from the group consisting of H— , HO— , a mono-phosphate, a di-phosphate and a tri-phosphate. Sig is defined in claim 596 as being covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y, z, and a combination thereof. Furthermore, Sig comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when so attached to the phosphate or when the modified nucleotide is incorporated into the oligo- or polydeoxynucleotide or when the oligo- or polydeoxynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof. Claims 597-616 depend from claim 596 and they define various narrower embodiments, including the nature of Sig (claims 597-598 and 606-611); covalent attachment of Sig (claims 599 and 612); the chemical linkage (claims 599-604); the nature of x, y and/or z (claim 605 and 613-614); the inclusion of at least one ribonucleotide (claim 615); and a structural formula for the claimed oligo- or polydeoxyribonucleotide (claim 616).

(i) I believe that claims 677-696 differ from claims 596-616 in the following respects. First, independent claim 677 does not recite "non-polypeptide" for the non-radioactive label moiety Sig. Second, specific members for Sig are recited in claim 677 (biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, a hapten and a combination). Dependent claims 678-696 are directed to various embodiments, such as the nature of Sig (claims 678-679 and 687-691); the covalent attachment of Sig (claims 680 and 692); the chemical linkage (claims 681-685); the elements x, y and/or z (claims 686 and 693-694); the inclusion of at least one ribonucleotide (claim 695); and the structural formula of the oligo- or polydeoxyribonucleotide (claim 696).

(ii) I also believe that claims 755-774 differ from claims 596-616 and 677-696 described above. First, claim 755, an independent claim, recites that Sig is covalently attached to PM through a chemical linkage comprising a polypeptide or a protein. Second, for the non-radioactive label moiety Sig, claim 755 lacks the recitation of the term "non-polypeptide" and the various members of Sig. Dependent embodiments are provided in claims 756-774 and include the nature of Sig (claims 756-757 and 761-766); the covalent attachment of Sig (claims 758 and 770); the chemical linkage (claims 759 and 767-769); the elements x, y and/or z (claims 760 and 771-772); the inclusion of at least one ribonucleotide (claim 773); and a structural formula for the claimed oligo- or polydeoxyribonucleotide (claim 774).

C. I believe that another aspect of the Engelhardt invention is defined in claims 617-636. In claim 617, the oligo- or polynucleotide is also complementary to a nucleic acid of interest or a portion thereof, and it comprises at least one modified nucleotide having the formula

Sig – PM – SM – BASE

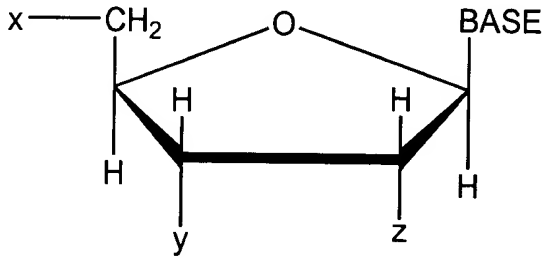
wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof. PM is attached to SM, BASE is attached to SM, and Sig is covalently attached to PM directly or via a chemical linkage. Sig comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when the modified nucleotide is incorporated into the oligo- or polynucleotide, or when the oligo- or polynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof, provided that when the oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3′ position of a terminal ribonucleotide, the chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3′ terminal ribonucleotide previously attached to the oligoribonucleotide or polyribonucleotide. Claims 618-636 are dependent embodiments and they include: the nature of Sig (claims 618-619 and 627-632); the covalent attachment of Sig (claims 620 and 633); the chemical linkage (claims 621-625); the nature of PM (claim 626); the nature of SM (claims 634-635); and the inclusion of at least one deoxyribonucleotide (claim 636).

(i) I believe that claims 697-715 are different from claims 617-636 in two respects. First, independent claim 697 does not recite the term "non-polypeptide" for the non-radioactive label moiety Sig. Second, claim 697 lists specific Sig members (biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, a hapten and a

combination). Other various aspects are given in claims 698-715 including the nature of Sig (claims 698-699 and 707-711); the covalent attachment of Sig (claims 700 and 712); the chemical linkage (claims 701-705); the nature of PM (claim 706); the nature of the sugar moiety (claims 713-714); and the inclusion of at least one deoxyribonucleotide (claim 715).

(ii) My review also shows that claims 775-793 differ from the afore-described claims 617-636 and 697-715 as follows. Unlike its counterparts (claims 617 and 697), independent claim 775 recites that Sig is covalently attached to PM via a chemical linkage comprising a polypeptide or a protein. Moreover, claim 775 does not recite the term "non-polypeptide" or the members of the non-radioactivel label moiety Sig, unlike claims 617 and 697. Dependent embodiments of claim 775 are given in claims 776-793. These embodiments include the nature of Sig (claims 776-777 and 781-785); the covalent attachment of Sig (claims 778 and 790); the chemical linkage (claims 779 and 787-789); the nature of PM (claim 780); the nature of SM (claims 791-792); and the inclusion of at least one deoxyribonucleotide (claim 793).

D. My review also shows me that another composition claimed in the Engelhardt application is an oligo- or polynucleotide as set forth in claims 637-657. As given by claim 637, this claimed composition is complementary to a nucleic acid of interest or a portion thereof, such oligo- or polynucleotide comprising at least one modified nucleotide having the structural formula:



BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and it is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine. The elements x, y and z are each selected from the group consisting of H— , HO— , a mono-phosphate, a di-phosphate and a tri-phosphate. Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y and z, and a combination thereof. Sig also comprises a non-polypeptide, non-radioactive label moiety which can be directly or indirectly detected when so attached to the phosphate or when the modified nucleotide is incorporated into the oligo- or polynucleotide, or when the oligo- or polynucleotide is hybridized to

the complementary nucleic acid of interest or a portion thereof. It is provided in the language of claim 637 that when the oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, the chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to the oligoribonucleotide or polyribonucleotide. Claims 638-657 are dependent embodiments directed to the nature of Sig (claims 638-639 and 647-652); the covalent attachment of Sig (claims 640 and 653); the chemical linkage (claims 641-645); the nature of elements x, y and/or z (claims 646 and 654-655); the inclusion of at least one deoxyribonucleotide (claim 656); and the structural formula for the oligo- or polynucleotide (claim 657).

(i) Based on my review, I also believe that claims 716-735 differ from claims 637-657 as follows. Claim 716, an independent claim, does not recite the term "non-polypeptide" for the non-radioactive label moiety Sig. Further, specific Sig members are listed in claim 716 (biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, a hapten and a combination). Other embodiments are given in claims 717-735, including the nature of Sig (claims 717-718 and 726-730); the covalent attachment of Sig (claims 719 and 731); the chemical linkage (claims 720-724); the nature of x, y and/or z (claims 725 and 732-733); the inclusion of at least one deoxyribonucleotide (claim 734); and the structural formula of the oligo- or polynucleotide (claim 735).

(ii) Claims 794-813 are different from claims 637-657 and 716-735 described above. Independent claim 794 recites that Sig is covalently attached to PM via a chemical linkage comprising a polypeptide or a protein. Unlike claims 637 and 716, claim 794 lacks the recitation for "non-polypeptide" and the various members for the non-radioactive label moiety Sig. Claims 795-813 depend from claim 794 and provide other embodiments including the nature of Sig (claims 794-795 and 800-805); the covalent attachment of Sig (claims 797 and 809); the chemical linkage (claims 798 and 806-808); the nature of PM (claim 799); the elements x, y and/or z (claims 799 and 810-811); the inclusion of at least one deoxyribonucleotide (claim 812); and a structural formula for the claimed oligo- or polynucleotide (claim 813).

E. As I indicated in Paragraph 9 above, several dependent claims (814-825) define embodiments wherein the non-radioactive label moiety Sig is attached indirectly to the phosphate moiety through a polypeptide or protein chemical linkage. Thus, such dependent claims 814, 817,

820 and 823 recite that the non-radioactive label moiety Sig is covalently attached to PM (or to at least one phosphate) through a chemical linkage comprising a polypeptide or a protein. In turn, claims 815, 818, 821 and 824 define the polypeptide as comprising polylysine. Other dependent claims (816, 819, 822 and 825) define such polypeptide or protein as being selected from avidin, streptavidin and anti-hapten immunoglobulin.

7. I read that in the November 26, 2001 Office Action, ten rejections (Paragraphs 7-9 & 11-17 in the Office Action) were raised against the former claims 454-567. Six of the ten rejections in the Office Action (Rejections Nos. 1 and 6-10; Paragraphs 7 and 13-17) concerned Ward et al., U.S. Patent No. 4,711,955 ("the Ward '955 Patent"). Of those six rejections involving the Ward '955 Patent, the first rejection was based on anticipation by the Ward '955 Patent and the last rejection was based on obviousness over the Ward '955 Patent in view of Falkow et al., U.S. Patent No. 4,358,535 ("the Falkow '535 Patent"). The first and last rejections are quoted below:

> **The First Rejection (Anticipation)[5]**
>
> Ward teaches modified nucleotides and methods of using and preparing the same. Specifically, Ward teaches the production and use of nucleic acid probes comprising a general structure (see abstract),
>
> "wherein B represents a purine, 7-deazapurine, or pyrimidine moiety covalently bonded to the C1'-position of the sugar moiety, provided that when B is purine or 7-deazapurine, it is attached at the N9-position of the purine or 7-deazapurine and when B is pyrimidine, it is attached at the N1-position, wherein A represents a moiety (i.e. Sig) consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, or DNA-RNA hybrid; wherein the dotted line represents a chemical linkage joining B and A, provided that if B is purine, the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine and wherein each of x, y and z represents (H-, HO-, etc. see abstract) either directly or when incorporated into oligo- and polynucleotides, provide probes which are widely useful." (see abstract).
>
> It is noted that the claims of the instant invention are broadly drawn to oligo- or polydeoxynucleotides or polyribonucleotides, wherein the Sig is covalently

[5] **Claims 454-461, 463-474, 476-489, 491-502, 504-518, 520-531, 533-546, 548-559 and 561-567 were rejected for anticipation by the Ward '955 Patent.**

attached to the PM (or x, y or Z) directly ***or through a chemical linkage***. Ward teaches the covalent attachment of the Sig to the PM through a chemical linkage (i.e. through the linkage of the sugar (SM) and the base (BASE) moieties – see abstract), therefore, Ward teaches the instant claims 454, 482, 510, 511, 539 and 567, and claims 457, 485, 514, and 542.

With respect to claims 455-56, 458, 463-474, 476-481, 483-484, 486, 491-502, 504-509, 512-513, 520-531, 533-538, 540-541, 543, 548-559, 561-566 the reference teaches that the probe (for general formula, see abstract) comprising the Sig can be a self-signalling moiety such as biotin, fluorescent dyes, electron-dense reagents (such as ferritin, colloidal gold, ferric oxide) or enzymes (such as peroxidase, alkaline phosphatase) (col. 18, 9-68).

With respect to claims 459-461, 487-489, 516-518, and 544-546, Ward teaches:

"the chemical linkages may include *any of the well known bonds including carbon-carbon single bonds, carbon-carbon double bonds, carbon-nitrogen single bonds, or carbon-oxygen single bonds*. However, it is generally preferred that *the chemical linkage include an olefinic bond at the .alpha.-position relative to B*. The presence of such an .alpha.-olefinic bond serves to hold the moiety A away from the base when the base is paired with another in the well known double helix configuration. This permits interaction with polypeptide to occur more readily, thereby facilitating complex formation. Moreover, single bonds with greater rotational freedom may not always hold the moiety sufficiently apart from the helix to permit recognition by and complex formation with polypeptide. *It is even more preferred that the chemical linkage group be derived from a primary amine, and have the structure --CH.sub2--NH--,* since such linkages are easily formed utilizing any of the well known amine modification reactions. *Examples of preferred linkages derived from allylamine and allyl-(3-amino-2-hydroxyl-1-propyl) ether groups have the formulae --CH.dbd.CH--CH.sub2--NH-- and ##STR12## respectively*.

Although these linkages are preferred, others can be used, including particularly olefin linkage arms with other modifiable functionalities, such as thiol, carboxylic acid, and epoxide functionalities.

This rejection could be overcome by amending the claims by deleting the recitation *"or through a chemical linkage"*.

[November 26, 2001 Office Action, pages 4-5; emphasis in the original]

The Tenth Rejection (Obviousness)[6]

The teachings of Ward are presented above. Specifically, Ward teaches the probe comprising Sig–PM–SM–BASE, wherein the covalent attachment of the Sig to the PM through a chemical linkage (i.e. through the linkage of the sugar (SM) and the base (BASE) moieties – see abstract). Ward also teaches that a variety of Sig labels (such as peroxidase and alkaline phosphatase) can be used in detection (col.

[6] Claims 462, 490, 519, and 547 were rejected for obviousness as being unpatentable over the Ward '955 Patent, as applied to claims 454-461, 463-474, 476-489, 491-502, 504-518, 520-531, 533-546, 548-559 and 561-567 above, and further in view of Falkow et al., U.S. Patent No. 4,358,535.

> 18, ln. 24-28), but does not teach the Sig label comprising a glycosidic linkage (i.e. using a Sig comprising a glycosidase).[7]
>
> The teachings of Falkow are presented above. Specifically, Falkow teaches enzymes of interest as labels (i.e. Sigs) include hydrolases, particularly esterases and glycosidases (i.e. which would be attached to PM via a glycosidic linkage), or oxidoreductases, particularly peroxidases (col. 4, ln. 12-14).
>
> In view of the teachings of Falkow, it would have been obvious to one of ordinary skill in the art at the time the invention was made to have modified the compound of Ward to include to a Sig comprising a glycosidic linkage (i.e. a Sig comprising a glycosidase), instead of a Sig (such as peroxidase, utilizing a different linkage), so as to have achieved an equally effective compound for nucleic acid detection. [November 26, 2001 Office Action, pages 18-19; footnote added]

8. As Enzo's scientific consultant, I am making this Declaration in support of the novelty and nonobviousness of the subject matter claimed in the Engelhardt application. I have been advised that my Declaration will be submitted to the U.S. Patent Office as part of a response that will supplement the May 28, 2002 Reply Under 37 C.F.R. §1.111. I am being compensated by Enzo for making this Declaration on its behalf.

9. Based upon my own training, background and experience, I would submit that at the time this application was first filed in June 1982, a person of ordinary skill in the art relevant to the subject matter being claimed, including nucleic acid chemistry and modification, which would include the attachment of labels and linker arms to nucleotides and nucleic acids, would have possessed or could have been actively pursuing an advanced degree in organic chemistry and/or biochemistry. Such an ordinarily skilled person could also be at least approaching the level of a junior faculty member with 2-5 years of relevant experience, or would at least be a postdoctoral student with several years of experience. I consider

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[7] There appears to be some confusion over the terms "glycosidic linkage" and "glycosidase." The former is a sugar chemical arm used for attaching a group to another molecule; the latter is an enzyme that hydrolyzes glycosidic bonds. The terms are not interchangeable, particularly with respect to the present dependent claims which call for a *glycosidic* linkage (chemical arm) and *not* a glycosidase (enzyme).

myself to possess the level of skill and knowledge of a person of at least ordinary skill in the art to which the present application and invention pertains.

The Engelhardt Invention Is Not Disclosed In The Ward '955 Patent Nor Is It Suggested By The Ward '955 Patent In Further View of The Falkow '535 Patent.

10. As a person of ordinary skill in the art, it is my opinion and conclusion that the invention in the Engelhardt application as set forth in the claims to be submitted to the Patent Office is novel over the disclosure of the Ward '955 Patent. Further, it is my opinion and conclusion that the invention claimed in the Engelhardt application would not have been obvious over the disclosure of the Ward '955 Patent in view of the Falkow '535 Patent disclosure.[8] My reasons are set forth in the following paragraphs.[9]

11. Before addressing the anticipation and obviousness rejections, I would like to provide some background to the Ward patents (including the '955 Patent cited in the November 26, 2001 Office Action) and the state of the art relating to the Engelhardt application and invention.

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[8] In a very narrow and strictly technical sense, I am willing to recognize the Examiner's position is his assertion that there is a chemical linkage between the phosphate moiety and the base moiety, and by extension, to a Sig moiety attached to a base moiety. Using the Examiner's reasoning to reject the Engelhardt claims, however, contradicts the way modifications of nucleotides (and nucleic acids) are classified in the chemical literature. These modifications are normally classified as being made to the base, to the sugar, or to the phosphate, the three chemically distinct components of a nucleotide. Chemists do not consider attaching a label to a sugar residue as either a phosphate modification or a base modification. Using the Examiner's logic, it could be argued that a reagent that is specific for say, the modification of cytosine, is really not specific to cytosine because cytosine is linked to all other bases in the nucleic acid polymer. As such, modifying cytosine would constitute a modification of all other bases linked to the modified cytosine bases. That position is not accepted in the art because it would throw nucleic acid nomenclature into disarray.

[9] Any opinions and conclusions given in this Declaration are done in light of my training, background and experience as one of at least ordinary skill in the art.

A. Prior to 1981 and the April 17, 1981 filing of the application leading to the Ward family of patents[10], the field of nucleic acid technology was still very much confined to the use of isotopic labels such as ^{32}P, ^{14}C and ^{3}H, or to heavy metal ions. The use of radioisotopes and heavy metals, such as mercury, is fraught with disadvantages, largely due to their hazardous nature. Some of the more obvious disadvantages are problems in disposal, health and safety considerations, and stability, i.e., the short shelf life of the radioisotopes. While at Yale and later at UVA, I worked with Drs. Ward and Langer (now Langer-Safer) on synthesizing non-isotopically labeled nucleotide analogs for use in making nucleic acid probes. We demonstrated that nucleotides could be modified and labeled non-radioactively in the non-disruptive base positions such that the non-radioactively labeled nucleotides could be incorporated into oligo- or polynucleotides. We confirmed that oligo- and polynucleotides containing such non-radioactively labeled nucleotides could be easily detected.

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[10] A paper was published subsequently to the April 17, 1981 filing of the first Ward application. Co-authored by the very same three persons named as inventors on the Ward '955 Patent (Pennina R. Langer, Alex A. Waldrop, and David C. Ward), our 1981 paper was titled "Enzymatic synthesis of biotin-labeled polynucleotides: Novel nucleic acid affinity probes" and it was published in Proc. Natl. Acad. Sci. 78:6633-6637 (November 1981). Our PNAS paper reported on the synthesis of nucleotide analogs, biotin-labeled dUTP and UTP, in which a biotin molecule was covalently bound to the C-5 position of the pyrimidine ring through an allylamine linker arm. We also reported that

> **"[t]hese biotin-labeled nucleotides are efficient substrates for a variety of DNA and RNA polymerases *in vitro*. Polynucleotides containing low levels of biotin substitution (50 molecules or fewer per kilobase) have denaturation, reassociation, and hybridization characteristics similar to those of unsubstituted controls. Biotin-labeled polynucleotides, both single and double stranded, are selectively and quantitatively retained on avidin-Sepharose, even after extensive washing with 8 M urea, 6 M guanidine hydrochloride, or 99% formamide. In addition, biotin-labeled polynucleotides can be selectively immunoprecipitated in the presence of antibiotin antibody and *Staphylococcus aureus* protein A. The unique features of biotin-labeled polynucleotides suggest that they will be useful affinity probes for the detection and isolation of specific DNA and RNA sequences."**
>
> **[Langer et al., abstract]**

A copy of Langer et al. is attached to my Declaration as Exhibit 2.

B. The principles or criteria behind our research that led to the issuance of the Ward '955 Patent[11] are set forth in the specification, column 6, line 36, through column 7, line 17:

> Several essential criteria must be satisfied in order for a modified nucleotide to be generally suitable as a substitute for a radioactively-labeled form of a naturally occurring nucleotide. First, the modified compound must contain a substituent or probe that is unique, i.e., not normally found associated with nucleotides or polynucleotides. Second, the probe must react specifically with chemical or biological reagents to provide a sensitive detection system. Third, the analogs must be relatively efficient substrates for commonly studied nucleic acid enzymes, since numerous practical applications require that the analog be enzymatically metabolized, e.g., the analogs must function as substrates for nucleic acid polymerases. For this purpose, probe moieties should not be placed on ring positions that sterically, or otherwise, interfere with the normal Watson-Crick hydrogen bonding potential of the bases. Otherwise, the substituents will yield compounds that are inactive as polymerase substrates. Substitution at ring positions that alter the normal "anti" nucleoside conformation also must be avoided since such conformational changes usually render nucleotide derivatives unacceptable as polymerase substrates. Normally, such considerations limit substitution positions to the 5-position of a pyrimidine and the 7-position of a purine or a 7-deazapurine.
>
> Fourth, the detection system should be capable of interacting with probe substituents incorporated into both single-stranded and double-stranded polynucleotides in order to be compatible with nucleic acid hybridization methodologies. To satisfy this criterion, it is preferable that the probe moiety be attached to the purine or pyrimidine through a chemical linkage or "linker arm" so that it can readily interact with antibodies, other detector proteins, or chemical reagents.
>
> Fifth, the physical and biochemical properties of polynucleotides

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[11] As described in Section 2 above, the Ward '955 Patent was but one of four U.S. patents that issued in the patent family. As listed on page 3 above, U.S. Patents Nos. 5,328,824; 5,449,767; and 5,476,928 were also issued. European Patent Nos. 0 063 879 B1 and 0 329 198 B1. were also granted as foreign counterparts.

containing small numbers of probe substituents should not be significantly altered so that current procedures using radioactive hybridization probes need not be extensively modified. This criterion must be satisfied whether the probe is introduced by enzymatic or direct chemical means.

Finally, the linkage that attaches the probe moiety should withstand all experimental conditions to which normal nucleotides and polynucleotides are routinely subjected, e.g., extended hybridization times at elevated temperatures, phenol and organic solvent extraction, electrophoresis, etc.

All of these criteria are satisfied by the modified nucleotides described herein.

C. The literature accorded recognition to our discovery with some authors crediting or characterizing the work of our group as a "landmark" for making the *first practical* non-radioactive nucleic acid probes.

(i) Symons provides the following description in Chapter 2 of his book Nucleic Acid Probes[12]:

> ***A landmark in the development of methods for labeling nucleic acid probes by nonisotopic means was the publication of Langer et al.[1] describing the synthesis of biotinylated nucleotides and their enzymatic incorporation into DNA and RNA***. The biotin was attached via a linker arm to the 5-position of the pyrimidine ring of dUTP (Figure 1) or UTP. Such nucleotides could be incorporated by the respective polymerases into DNA or RNA. . . .
>
> [Nucleic Acid Probes, page 35; emphasis added]

(ii) In their book DNA Probes[13], Keller and Manak provide the following description:

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[12] **The full citation is Robert H. Symons, Ph.D., Nucleic Acid Probes, CRC Press, Inc., Boca Raton, Florida, 1989, Chapter 2 ("Preparation and Detection of Nonradioactive Nucleic Acid and Oligonucleotide Probes"), page 35. A copy of page 35 from Symons's book is attached as Exhibit 3.**

[13] **The full citation is George H. Keller and Mark M. Manak, DNA Probes, Stockton Press, New York, 1989, Section Four: Non-Radioactive Labeling Procedures, page 107. A copy of page 107 from Keller and Manak's book is attached as Exhibit 4.**

> ***The first non-radioactive DNA probes, of a practical design, were described in the scientific literature by Langer et al. (1981).*** This early probe labeling scheme employed biotin-labeled deoxyribonucleotide triphosphates, incorporated into the probe DNA by enzymatic polymerization. The modified nucleotides, in turn, were developed as the result of years of experimentation with mercurated nucleotides and polynucleotides (Dale and Ward, 1975; Dale *et al.*, 1973; Dale *et al.*, 1975). The most widely used modified nucleotide is biotin-11-dUTP, as shown in Figure 4.1.
>
> The molecule incorporates the following features: modification at the C-5 position where it will not interfere with hydrogen bonding, a double bond to minimize flexing of the linker arm and a linker arm long enough to ensure access of detection reagents to the biotin. . .
>
> [DNA Probes, page 107; emphasis added]

12. Having provided some background to the Ward patents, I now wish to address the first rejection (anticipation) from the November 26, 2001 Office Action, as quoted in Section 7 above.

The First Rejection (Anticipation)

(i) With respect to claims 454, 482, 510, 511, 539 and 567, and claims 457, 485, 514 and 542, it is my opinion and conclusion as a person of ordinary skill in the art that the Ward '955 Patent does not disclose the subject matter of these claims. As indicated in Section 11.B above, the Ward '955 Patent limits the attachment of the non-radioactive labels to the non-disruptive base positions of the pyrimidine, purine or deazapurine, namely, the 5-, 8- and 7-positions, respectively. The claims in the Engelhardt application are directed to compositions in which the non-polypeptide, non-radioactive label moiety Sig is attached to the phosphate moiety -- which is not to the base, and certainly is not to any of the aforementioned Ward positions (the 5-, 8- or 7-positions of a pyrimidine, purine or deazapurine, respectively). The Examiner stated on page 4 in the November 26, 2001 Office Action that:

> Ward teaches the covalent attachment of the Sig to the PM through a chemical linkage (i.e. through the linkage of the sugar (SM) and the base (BASE) moieties – see abstract), therefore, Ward teaches instant claims 454, 482, 510, 511, 539, and 567, and claims 457, 485, 514, and 542.

As a person of ordinary skill in the art, it is my opinion and conclusion that it is incorrect and improper to characterize the specific base labeling positions in the Ward '955 Patent for attaching non-radioactive labels as being indirectly attached to the phosphate moiety through the linkage of the sugar. The basis for my foregoing statement is set forth in the following several paragraphs.

(a) The sugar is a distinct element of a nucleotide and it is not recognized in the art to my knowledge as being an indirect linkage of the phosphate moiety to the base moiety. That a nucleotide is recognized as having three distinct elements (a sugar, a phosphate and a base) is evidenced by several definitions and descriptions quoted below which have been culled from various dictionaries, glossaries and science textbooks.

> (a)
> A nucleotide (Fig. 1-1) has three components: (i) a purine or pyrimidine <u>base</u>, linked through one of its nitrogens by an N-glycosidic bond to (ii) a 5-carbon cyclic <u>sugar</u> (the combination of base and sugar is called a nucleoside) and (iii) a <u>phosphate</u>, esterified to carbon 5 of the sugar. . . .
>
> [<u>DNA Synthesis</u>, Arthur Kornberg, W. H. Freeman and Company, San Francisco, 1974, page 3; copy attached as Exhibit 5; underline in the original]

(b)
COMPONENTS OF NUCLEIC ACIDS
Nucleotides consist of three components: a pyrimidine or purine base linked to a sugar, either ribose or deoxyribose, and phosphate esterified to the sugar at carbon 2, 3, or 5. Esterification at carbon 5 is most common.

[Principles of Biochemistry, Sixth Edition, White et al., McGraw-Hill Book Company, New York, 1978; page 167 copy attached as Exhibit 6]

(c)
NUCLEOTIDES, THE BUILDING BLOCKS OF NUCLEIC ACIDS
There are two chemically different types of nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Both DNA and RNA contain four different nucleotides. Each *nucleotide* contains a nitrogenous base known as a *purine* or a *pyrimidine*; a sugar, *ribose* in RNA; *deoxyribose* in DNA; and a phosphoryl group. . . .

[Biochemistry, Geoffrey Zubay, Addison-Wesley Publishing Company, 1983, page 661; copy attached as Exhibit 7; emphasis in the original]

(d)
nucleotide (Levene 1909) – any of the monomeric units which are the building blocks of the polynucleotides referred to as → nucleic acids (Table 10). A n. [nucleotide] is a phosphate ester of the N-glycoside of a nitrogenous base and consists of a purine or pyrimidine base, a pentose (D-ribose in → ribonucleic acid, 2'-deoxy-D-ribose in → deoxyribonucleic acid) and a phosphate (PO_4) group. . . .

[Glossary of Genetics (Classical and Molecular), Fifth Edition, Rieger et al., Springer-Verlag, Berlin and New York, 1991; copy attached as Exhibit 8]

(b) It should not be overlooked that the three elements making up a nucleotide (sugar, phosphate and base) are not only different structurally, but they are different chemically, such that these elements are subject to *different* chemical reactions.

(1) The reactions of the sugar moiety and the base were described in a textbook more than twenty-five years ago. In Nucleic Acid Structure An Introduction[14], Guschlbauer provides the following description:

> **3.2 Chemical Reactions of Nucleic Acids and Their Constituents**
> **3.2.1 REACTIONS OF THE SUGAR MOIETY**
> **Periodate reaction.** One of the most widely employed reactions in RNA chemistry is the periodate oxidation of the *cis*-glycol of ribose (2' and 3'-OH). No reaction occurs with deoxyribose or arabinose. This reaction yields the terminal nucleoside as 2',3'-dialdehyde and is useful in identifying terminal nucleosides in RNA and/or coupling them with other reagents.
>
> **Color Reactions.** Two very old methods, which are still widely used, depend on the hydrolysis of the glycosidic linkage of purine nucleosides by hot concentrated acids, with the subsequent transformation of the sugar into furfural derivatives. These derivatives, in turn, give specific color reactions with such aldehyde specific reagents as orcinol, which reacts with furfural (from ribose) and ferric chloride to give a green color; the intensity of this color is proportional to the ribose concentration. A blue color is obtained with deoxyribose in concentrated sulfuric acid with diphenylamine (7).
>
> **3.2.2 CHEMICAL REACTIONS OF BASES**
> A very large number of reactions, described in the literature, imply the modification of the nucleic acid bases. A few of the most widely useful and widely used ones are described.
>
> **Nitrous acid.** Sodium nitrite at low pH reacts with aromatic amines to form phenols. This reaction has been widely used to induce chemical mutations (see Chapter 7). Since the phenolic forms of the bases are not stable, the corresponding keto compounds are obtained:
>
> Ade → Hyp, Gua → Xan, Cyt → Ura.
>
> The reaction rate decreases in the order Cyt > Ade > Gua. Furthermore, exposed amino groups are more susceptible to attack than those involved in a structure or H-bonds. Sodium nitrite, therefore, acts more efficiently on single-stranded nucleic acids than on double-stranded ones.
>
> Dilute nitrous acid attacks Ura to yield 5-nitrouracil, which has a characteristic UV spectrum (peak at 350 nm).
>
> **Formaldehyde.** Formaldehyde (like many other aldehydes or ketones) reacts with the amino groups of free bases or single-stranded nucleic acids. Since it can react with accessible amino groups, formaldehyde is used to measure the kinetics of nucleic acid unwinding (8). The chemistry is not well understood (possibly a Schiff's base as an intermediate). There is a detailed review by Feldman (9) on the subject.

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[14] The full citation is Wilhelm Guschlbauer, Nucleic Acid Structure: An Introduction, Springer-Verlag, New York, NY, 1976, Section 3.2 Chemical Reactions of Nucleic Acids and Their Constituents, pages 24-26. A copy of pages 24-26 is attached as Exhibit 9.

Hydroxylamine. Hydroxylamine is a strong mutagen and reacts specifically with Cyt and hom^{5}Cyt (5-hydroxy-methyl-cytosine); but it also reacts less strongly with Thy and Ura. The former reaction is probably a two-step one, the first step being the addition of NH_2OH on the C^5-C^6 double bond, then the elimination of two moles of ammonia, and water, and finally, the formation of Ura.

Halogenation. Like many compounds, nucleic acid bases are attacked by halogens, the pyrimidines at position 5, the purines at position 8. Halogenobases have found wide application in chemotherapy (see Chapter 7), br^{5}dUrd is a powerful dThd analogue; br^{3}Ado abd br^{3}Guo have been used in RNA polymerase studies.

Alkylation. Dimethylsulfate (or other alkylating agents) act on nucleosides and nucleic acids to yield alkylated bases, which are frequently powerful mutagens or carcinogens. The alkylation site is generally that of protonation or amino groups, i.e., N^7 (and N^1) in gua and Ino, N^1 and N^6 in Ade, N^3 and N^4 in Cyt. Since they frequently substitute at the site where hydrogen bonds are formed, aberrant pairings are expected. Bi-functional alkylating agents, like nitrogen mustards ($Cl–CH_2–CH–CH_2–NR–CH_2–CH_2–Cl$), or some antibiotics, like mitomycin, can induce cross-links between two strands of DNA by reacting with two Gua on opposite strands.

Other reactions. The reaction with P_2S_5 permits the obtention of sulfur-substituted bases, which are useful intermediates. These mercaptols (with a sulfur rather than a keto group substituent) can be treated with alcoholic ammonia to yield amines.

The literature on the chemistry of nucleosides and nucleotides is enormous. Several classics exist on this subject (e.g., Ref. 10).

(2) Reactions involving the phosphate moiety were earlier described by Brown in Basic Principles in Nucleic Acid Chemistry.[15] Phosphate reactions include, for example, hydrolysis, elimination and alkylation.

(c) I know of no publication or commonly accepted characterization in the nucleic acid arts that describes the attachment of labels or even the modification of the base to be an indirect attachment or modification to

[15] **The full citation is Brown, D. M., "Chemical Reactions of Polynucleotides and Nucleic Acids," Chapter 1 in Basic Principles in Nucleic Acid Chemistry, Volume II, Paul O. P. Ts'O (Editor), Academic Press, Inc., New York and London, 1974. Section IV of Brown's chapter covers pages 38-57 and is titled "Reactions Affecting the Internucleotide Linkage." A copy of pages 38-57 from Brown's chapter is attached as Exhibit 10.**

the phosphate moiety through the chemical linkage of the sugar moiety. It is my opinion and conclusion that even if there were a publication describing the attachment or modification to the base to be an indirect attachment or modification to the phosphate moiety through the chemical linkage of the sugar moiety, such would not be a description or characterization that is commonly accepted in the art. As discussed in the preceding paragraph (b), a person of ordinary skill in the art would appreciate that the three elements of a nucleotide (sugar, phosphate and base) are distinct elements with different chemical properties and different chemical reaction specificities. Furthermore, from a reading of the Engelhardt specification, a person of ordinary skill in the art would understand that the sugar moiety, already a part of the nucleotidyl and nucleic acid structure, is not a chemical linkage as it is commonly defined in the art and also disclosed in the Engelhardt specification. To illustrate, I have attached DNA and RNA structures as Exhibits 11 and 12, respectively. As shown in both structures, to characterize the Ward patent as teaching the covalent attachment of the Sig to the PM through a chemical linkage (i.e. through the linkage of the sugar (SM) and the base (BASE) moieties is, in my opinion as a person of ordinary skill in the art, incorrect for at least two significant reasons. First, the characterization given in the November 26, 2001 Office Action (page 4, last full paragraph) requires that *both* the base and sugar be chemical linkages. It is my opinion and conclusion that the base and sugar are *not* chemical linkages in the context of the Engelhardt specification and claimed invention because the base and sugar are constituent elements of a nucleotide. Second, there are many phosphate, sugar and base moieties in a DNA or RNA chain (three are shown in each of Exhibits 11 and 12). To characterize any of the constituent nucleotidyl elements -- phosphate, sugar or base -- as Engelhardt-type (or Ward-type) chemical linkages, begs the question, in my opinion, as to where in the nucleotidyl structure a chemical group or label (Sig) is attached.

One investigator could say that the base is a chemical linkage to the sugar while another investigator could assert that the base and sugar are chemical linkages to the phosphate. Thus, the characterization that the base, or the base and sugar, are somehow chemical linkages to the phosphate in accordance with the Engelhardt specification and claimed invention, is confusing in light of conventional nucleic acid nomenclature.[16]

(ii) In view of the above paragraphs and exhibits (2-12), it is my opinion and conclusion that the Engelhardt claims are not disclosed in the Ward '955 Patent. As set forth above, the sugar, phosphate and base are separate and distinct elements in a nucleotide. Further, the natural sugar moiety already present in the nucleotide or polynucleotide is not an indirect chemical linkage of the base moiety to the phosphate moiety, as disclosed in the Engelhardt specification. Moreover, as different elements, the sugar, phosphate and base are subject to different chemical reactions.

13. I now wish to turn to the last rejection (obviousness) in the November 26, 2001 Office Action.

The Tenth Rejection (Obviousness)

(i) It is my opinion and conclusion that it would not have been obvious to one of ordinary skill in the art to have modified Ward's compound to include a glycosidic linkage instead of peroxidase, in view of the Falkow '535 Patent and its disclosure of enzymes as labels, and thereby arrive at the invention in the Engelhardt application. As indicated in Paragraph (12(i)) above, the Ward '955

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[16] Even with radioisotopic chemistry, the sugar, phosphate and base elements are viewed as separate nucleotidyl elements. For example, ^{32}P is described for labeling the phosphate moiety; ^{3}H and ^{14}C are described for labeling the sugar or base moieties.

Patent does not disclose among its compounds a non-polypeptide, non-radioactive label moiety Sig attached to the nucleotidyl phosphate moiety in an oligo- or polynucleotide.

(ii) As indicated above in Paragraph 6(A through H), the claims in the Engelhardt application recite a non-polypeptide, non-radioactive label moiety Sig, or the members of Sig comprise biotin, iminobiotin, an electron dense component, a magnetic component, a metal-containing component, a fluorescent component, a chemiluminescent component, a chromogenic component, a hapten or a combination of any of the foregoing. The element Sig in the Engelhardt claims is not an enzyme as disclosed in the Falkow '535 Patent. Thus, the addition of the Falkow '535 Patent does not provide the necessary disclosure which would have motivated or allowed a person of ordinary skill in the art to arrive at the claims in the Engelhardt application from a combined reading of the Ward '955 Patent and the Falkow '535 Patent (i.e., Ward in view of Falkow).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.

06/26/02
Date

Dr. Alexander A. Waldrop, III

* * * * * * *

Alex A. Waldrop, III

Born: December 26, 1948

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OBJECTIVES

To develop sensitive, easy to use, analytical methods for detecting biomolecules and environmentally important substances, especially using chemiluminescent and fluorescent methods. To develop or apply the methods of nucleic acid and protein chemistry to solve problems in molecular biology leading to advances in medicine or biotechnology.

ACHIEVEMENTS

Co-inventor of non-radioactively-labeled nucleotides, including biotinyl nucleotides (U.S. Patents Nos. 4,711,955; 5,328,824; 5,449,767; and 5,476,928). Co-inventor of activated 9-acridinecarboxylic acid chemiluminescent system. Experienced in chemistry of nucleic acids and proteins, especially the synthetic chemistry of nucleotides, peptides, and their oligomers, and in the chemistry of mercury, osmium, and palladium; familiar with NMR, UV-Visible, IR, and fluorescent spectroscopic techniques, and with TLC, HPLC, gel filtration, and ion exchange chromatographic procedures; experienced in the use of DNA polymerases and nucleases. Experienced in detection systems for nucleic acids, especially chemiluminescence. Experienced in chemistry of acridine and acridinium compounds. Experienced with several ELISA enzymes, including horseradish peroxidase (HRPO), alkaline phosphatase, glucose oxidase, and β–galactosidase.

EDUCATION

Ph.D. (Biophysics) 1977, Johns Hopkins University, Baltimore, Maryland.
B.S. (Chemistry) 1970, Magna cum Laude, University of Virginia.

EXPERIENCE

Founder and Principal Scientist, Started Company at Center for Environmental Enterprise (CEE), 2000 to present. Further characterized 9-Acridinecarbonylimidazole (AcriGlow 301) and its reaction with peroxide in various buffers and solvents. Examined ways of removing peroxide impurities from solvents, detergent and polymer solutions. Tested screening assay for detecting pollutants in environmental water samples. Served as consultant for Brims Ness, Maine Standards, Enzo Biochem, Inc.

Visiting Scientist, Maine Medical Center Research Institute, 1994 to 2000. Synthesized and characterized modified acridancarboxylic acid ester. Demonstrated substrate activity with HRPO. Invented and characterized activated 9-acridinecarboxylic acid derivatives. Demonstrated high sensitivity assay of glucose oxidase and alkaline phosphatase. HPLC of acridine derivatives. HPLC of synthetic oligonucleotides.

Research Scientist, IDEXX Laboratories, Inc., 1992 - 1993. Optimization of HRPO assay systems.

Staff Scientist, Gen-Probe, Inc., 1985 - 1992. Synthesized and designed acridinium esters. Helped design linker arms, optimize detection of acridinium esters, stabilize acridinium esters, improve elution of nucleic acids from solid supports. Characterized acridinium esters by HPLC, UV and chemiluminescence.

Research Associate, Department of Microbiology, University of Virginia, 1982 - 1985. Developed new DNA sequencing method similar to Sanger approach, but which leaves functional 3' ends, which can be ligated to produce a set of deletion mutants or can be extended under conditions forcing misincorporation to generate a set of point mutations. Synthesized series of 5'-thymidine triphosphate derivatives containing a 3'-phosphate mono-, di-, or triester group. Showed that these analogs were not substrates for T4 or Klenow DNA polymerase. Developed simple, rapid gel filtration method for purifying and desalting nucleotides. Synthesized an analog of dUTP containing an EDTA group and showed that it can be enzymatically incorporated into DNA.

Assistant Professor, Department of Chemistry, University of Virginia, 1980-1982. Prepared nucleotide derivatives of tubercidin. Characterized allylamine derivatives. Taught biophysical chemistry.

Postdoctoral Research Fellow, Department of Molecular Biophysics and Biochemistry (laboratory of Dr. David C. Ward), Yale University, 1977-1980. Synthesized modified pyrimidines to incorporate in vitro into nucleic acids, using reactions between heavy metals and nucleic acid components. Developed nucleotide analogs used for gene detection in situ. Biotinyl nucleotides now selling commercially.

Predoctoral Fellow, Department of Biophysics (laboratory of Dr. Michael Beer), Johns Hopkins University, Baltimore, Maryland, 1970-1977. Developed multiple heavy atom stains for electron microscopy of nucleic acids.

HONORS Echols Scholar, Phi Eta Sigma, Hugh Miller Spencer Scholarship in Chemistry, 1970.

MEMBER Alpha Chi Sigma, Sigma Xi, AAAS, AACC, American Chemical Society.

Alex A. Waldrop, III

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(3) Langer P.R., Waldrop, A.A., and Ward, D.C.; Enzymatic Synthesis of Polynucleotides Containing Biotin: Novel Nucleic Acid Affinity Probes, Proc. Natl. Acad. Sci. U.S.A., 78, 6633-6637 (1981).

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(5) Waldrop, Alex A., III; Fellers, Jonathan; Vary, Calvin P. H.; Chemilumminescent Determination of Hydrogen Peroxide with 9-Acridinecarbonylimidazole and Use in Measurement of Glucose Oxidase and Alkaline Phosphatase Activity, Luminescence 15(3), 168-182, (2000).

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(1) Ward, D.C., Langer, P.R., and Waldrop, A.A.; Modified Nucleotides and Methods of Preparing and Using Same, U.S. Patent 4,711,955 (December 8, 1987). (European Pat. Appl. EP 63879 A2)

(2) Arnold, Lyle J., Waldrop, Alex A., III, Hammond, Philip W.; Protected Chemiluminescent Labels, U. S. Patent # 4,950,613 (Aug. 21, 1990). (European Pat. Appl. EP 330433 A2).

(3) Ward, D.C., Langer, P.R., and Waldrop, A.A.; Methods of Using Labeled Nucleotides. U.S. Patent #5,328,824 (July 12, 1994).

(4) Ward, D.C., Langer, P.R., and Waldrop, A.A.; Modified Polynucleotides and Methods of Preparing Same. U.S Patent #5,449,767 (Sept.12, 1995).

(5) Ward, D.C., Langer, P.R., and Waldrop, A.A.; Modified Nucleotides and Polynucleotides and Complexes Form Therefrom. U.S Patent #5,476,928 (Dec.19, 1995).

(6) Arnold, Lyle, J., Jr.; Nelson, Norman C.; Reynolds, Mark A.; Waldrop, Alex A., III; Polycationic Supports and Nucleic Acid Purification, Separation and Hybridization. U. S. Patent #5,599,667 (Feb 4, 1997). (European Pat. Appl. EP 281390 A2).

(7) Waldrop, Alex A., III and Vary, C.P.H., Peroxide-Based Chemiluminescent Assays and Chemiluminescent Compounds Used Therein. Patent pending (Submitted 1997 as Provisional Patent Application).

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Biochemistry

Enzymatic synthesis of biotin-labeled polynucleotides: Novel nucleic acid affinity probes

(nucleotide analog/DNA and RNA polymerase/avidin-Sepharose/antibiotin antibody/immunoprecipitation)

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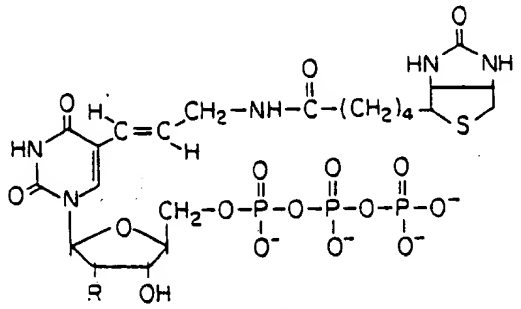
ABSTRACT Analogs of dUTP and UTP that contain a biotin molecule covalently bound to the C-5 position of the pyrimidine ring through an allylamine linker arm have been synthesized. These biotin-labeled nucleotides are efficient substrates for a variety of DNA and RNA polymerases *in vitro*. Polynucleotides containing low levels of biotin substitution (50 molecules or fewer per kilobase) have denaturation, reassociation, and hybridization characteristics similar to those of unsubstituted controls. Biotin-labeled polynucleotides, both single and double stranded, are selectively and quantitatively retained on avidin-Sepharose, even after extensive washing with 8 M urea, 6 M guanidine hydrochloride, or 99% formamide. In addition, biotin-labeled polynucleotides can be selectively immunoprecipitated in the presence of antibiotin antibody and *Staphylococcus aureus* protein A. The unique features of biotin-labeled polynucleotides suggest that they will be useful affinity probes for the detection and isolation of specific DNA and RNA sequences.

Nucleotide analogs that can function as indicator "probes" when incorporated in polynucleotides would be of significant utility in many procedures used in biomedical and recombinant DNA research. When used in conjunction with immunological, histochemical, or affinity detector systems, such reagents could provide suitable alternatives to radioisotopes for the detection, localization, and isolation of nucleic acid components. Biotin (vitamin H) has many features that make it an ideal probe candidate. The interaction between biotin and avidin, a 68,000-dalton glycoprotein from egg white, has one of the highest binding constants ($K_{dis} = 10^{-15}$) known (1). When avidin is coupled to appropriate indicator molecules (fluorescent dyes, electron-dense proteins, enzymes, or antibodies), minute quantities of biotin can be detected (2–8). The specificity and tenacity of the biotin–avidin complex has been exploited to develop methods for the visual localization of specific proteins, lipids, and carbohydrates on or within cells (for review, see ref. 2). Davidson and associates (9–11) chemically crosslinked biotin to RNA, via cytochrome *c* or polyamine bridges, and used these RNA–biotin complexes as probes for *in situ* hybridization. The sites of hybridization were visualized in the electron microscope through the binding of avidin-ferritin or avidin-methacrylate spheres. Although this approach to the detection of polynucleotide sequences was successful in the specialized cases examined, a simpler and more general procedure for preparing biotin-substituted nucleic acids was desirable. Biotin directly attached to a nucleotide that functions as an efficient polymerase substrate would be more versatile, both in the experimental protocols and in the detection methods that could be used.

We have synthesized a number of nucleotide analogs that contain potential probe determinants (e.g., biotin, iminobiotin, and 2,4-dinitrophenyl groups) covalently attached to the pyrimidine or purine ring in the hope that one of them might prove to be a useful affinity reagent. This report describes the synthesis of biotin-labeled derivatives of UTP and dUTP (1 and 2, respectively) that are substrates for RNA or DNA polymerases. The properties of the resulting biotin-substituted polynucleotides appear to satisfy the basic criteria required of a good affinity probe.



1, R = OH
2, R = H

MATERIALS AND METHODS

Materials. Standard NTPs were purchased from P-L Biochemicals, and dUTP was obtained from Sigma. Radiolabeled nucleotides were products of New England Nuclear or Amersham Radiochemicals. *Escherichia coli* DNA polymerase I, both holoenzyme and Klenow fragment, was obtained from Boehringer Mannheim; restriction enzymes were from New England BioLabs or Bethesda Research Laboratories. The following enzymes and reagents were gifts: T7 RNA polymerase and T7 DNA (J. Coleman); herpes simplex DNA polymerase (B. Francke); L1210 and HeLa cell DNA polymerases α and β (H. S. Allaudeen); avian myeloblastosis reverse transcriptase (RNA-dependent DNA nucleotidyltransferase) (S. Weissman); murine and calf thymus RNA polymerase II (R. Roeder); *E. coli* RNA polymerase (P. Farnam); and rabbit antibiotin serum (F. Harmon).

Synthesis of 1 (Bio-UTP) and 2 (Bio-dUTP). *Mercuration step.* The 5-mercurated derivatives of UTP and dUTP were prepared by a modification of the procedure of Dale *et al.* (12). UTP (570 mg, 1.0 mmol) or dUTP (554 mg, 1.0 mmol) in 100 ml of 0.1 M sodium acetate, pH 6.0, was treated with mercuric acetate (1.59 g, 5.0 mmol). The solution was heated at 50°C for 4 hr and then cooled on ice. Lithium chloride (392 mg, 9.0 mmol)

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Abbreviations: AA-UTP and AA-dUTP, 5-(3-amino)allyl uridine and deoxyuridine triphosphates, respectively; Bio-UTP and Bio-dUTP, 5-allylaminobiotin-labeled UTP and dUTP, respectively; Bio-RNA and Bio-DNA, biotin-labeled RNA and DNA, respectively; MVM, minute virus of mouse; RF, replicative form.

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was added, and the solution was extracted six times with equal volumes of ethyl acetate to remove excess $HgCl_2$. The efficiency of the extraction process was monitored by estimating the mercuric ion concentration in the organic layer by using 4,4'-bis(dimethylamino)-thiobenzophenone (13). The extent of nucleotide mercuration, determined spectrophotometrically by following the iodination of an aliquot of the aqueous solution (14), was routinely 90–100%. The nucleotide products in the aqueous layer, which often became cloudy during the ethyl acetate extraction, were precipitated by the addition of 3 vol of ice-cold ethanol and collected by centrifugation. The precipitate was washed twice with cold absolute ethanol and once with ethyl ether and then air dried. These products were used for the synthesis of the 5-(3-amino)allyl nucleotides without further purification.

Synthesis of 5-(3-amino)allyluridine and deoxyuridine 5' triphosphates (AA-UTP and AA-dUTP). Organomercurial compounds can be alkylated or arylated under mild conditions by reaction with olefins in the presence of a palladium catalyst (15). Bergstrom and associates (16, 17) have recently used this procedure for the synthesis of C-5-substituted pyrimidine nucleosides. We have also adopted this general synthetic approach for introducing the allylamine linker arm. The mercurated nucleotides were dissolved in 0.1 M sodium acetate, pH 5.0, and adjusted to 20 mM (A_{267}, 200 units/ml). A fresh 2.0 M solution of allylamine (Aldrich) was prepared by slowly adding 1.5 ml of allylamine (13.3 M) to 8.5 ml of ice-cold 4 M acetic acid. Three milliliters (6.0 mmol) of the neutralized allylamine stock was added to 25 ml (0.5 mmol) of nucleotide solution. One nucleotide equivalent of K_2PdCl_4 (163 mg, 0.5 mmol; Alfa–Ventron, Danvers, MA) in 4 ml of water was then added to initiate the reaction; the solution gradually turned black and metal (Hg and Pd) deposits appeared on the walls of the reaction vessel. After standing at room temperature for 18–24 hr, the reaction mixture was passed through a 0.45-μm membrane filter (Nalgene) to remove most of the remaining metal precipitate. The yellow filtrate was diluted 1:5 with H_2O and applied to a 100-ml column of DEAE-Sephadex A-25 (Pharmacia). After washing with 1 column vol of 0.1 M sodium acetate, pH 5.0, the products were eluted by using a 1-liter linear gradient (0.1–0.6 M) of sodium acetate, pH 8–9, or Et_3NHCO_3, pH 7.5. The desired product was in the major UV-absorbing peak, which eluted between 0.30 and 0.35 M salt. Because spectral analysis showed that this peak contained several products, final purification was achieved by reverse-phase high-pressure liquid chromatography on columns of Partisil-ODS2, using either 0.5 M $(NH_4)_3PO_4$, pH 3.3 (analytical separations), or 0.5 M Et_3NHOAc, pH 4.3 (preparative separations), as eluents. AA-UTP and AA-dUTP were the last peaks to elute from the column and they were cleanly resolved from three as-yet unidentified contaminants. The characterization of the (3-amino)allyl nucleotides by proton NMR, elemental, spectral, and chromatographic analyses will be presented in detail elsewhere. These studies clearly showed that the (3-amino)allyl substituent is attached to the C-5 position of the pyrimidine ring and that the olefinic protons are in the *trans* configuration.

Conversion of AA-UTP or AA-dUTP to Bio-UTP and Bio-dUTP. Biotinyl-N-hydroxysuccinimide ester was prepared from biotin (Sigma) as described (3). AA-UTP·$4H_2O$ (70 mg, 0.1 mmol) or AA-dUTP·H_2O (63 mg, 0.1 mmol) in 20 ml of 0.1 M sodium borate, pH 8.5, was treated with the ester (34.1 mg, 0.1 mmol) in 2 ml of dimethylformamide. The reaction mixture was left at room temperature for 4 hr and then loaded directly onto a 30-ml column of DEAE-Sephadex A-25 previously equilibrated with 0.1 M Et_3NHCO_3, pH 7.5. The column was eluted with a 400-ml linear gradient (0.1–0.9 M) of Et_3NHCO_3. Fractions containing bio-dUTP or bio-UTP, which eluted at 0.55–0.65 M Et_3NHCO_3, were desalted by rotary evaporation in the presence of methanol and then dissolved in water. Occasionally, a slightly cloudy solution was obtained: this turbidity, due to a contaminant in some Et_3NHCO_3 solutions, was removed by filtration through a 0.45-μm filter. For long-term storage, the nucleotides were converted to sodium salts by briefly stirring the solution in the presence of Dowex 50 (Na^+). After filtration, the nucleotide was precipitated by the addition of 3 vol of cold ethanol, washed with ethyl ether, dried at reduced pressure over sodium hydroxide pellets, and stored in a desiccator at −20°C. For immediate use, the nucleotide solution was made 20 mM in Tris·HCl, pH 7.5, and adjusted to a final nucleotide concentration of 5 mM. Stock solutions were stored at −20°C.

Analysis. Bio-dUTP: Calcd. for $C_{22}H_{30}N_5O_{19}P_3SNa_4 \cdot H_2O$: C, 29.80; H, 3.38; N, 7.89; P, 10.47; S, 3.61. Found: C, 30.14; H, 3.22; N, 7.63; P, 10.31; S, 3.70. Bio-UTP: Calcd. for $C_{22}H_{30}N_5O_{19}P_3SNa_4 \cdot 3\,H_2O$: C, 29.15; H, 3.19; N, 7.45; P, 9.89; S, 3.41. Found: C, 28.76; H, 3.35; N, 7.68; P, 9.81; S, 3.32. The spectral properties of bio-dUTP and bio-UTP at pH 7.5 [λ_{max} 289 nm (ε = 7100); λ_{max} 240 nm (ε = 10,700); λ_{min} 262 nm (ε = 4300)] reflect the presence of an exocylic double bond conjugated with the pyrimidine ring. These nucleotides also give a strong positive reaction (an orange-red color) when treated with *p*-dimethylaminocinnamaldehyde in ethanolic sulfuric acid, a procedure used for biotin quantitation (18). However, in contrast to AA-dUTP and AA-UTP, they do not give a positive ninhydrin reaction.

RESULTS

Bio-UTP and Bio-dUTP have been synthesized. These analogs were then tested for their ability to function as substrates for a series of purified nucleic acid polymerases *in vitro*. As shown in Fig. 1, Bio-dUTP is an excellent substrate for *E. coli* DNA polymerase I using either the nick-translation protocol of Rigby *et al.* (19) or the "gap-filling" reaction described by Bourguignon *et al.* (21). Although it is incorporated at an initial rate that is only 30–40% of that of the control reaction with TTP, the final specific activities (and the extent of polymerization) that can be achieved are essentially the same. Bio-dUTP is also a substrate for bacteriophage T4 DNA polymerase, DNA polymerases α and β from murine (A-9) and human (HeLa) cells, and the DNA polymerase of herpes simplex virus, with incorporation efficiencies similar to that of *E. coli* DNA polymerase I (not shown). In addition, Bio-dUTP will support DNA synthesis in a nuclear replication system prepared from baby hamster kidney cells infected with herpes simplex virus (unpublished data). In contrast, Bio-dUTP does not function as a substrate for avian myeloblastosis virus reverse transcriptase under standard incubation conditions using mRNA–oligo(dT), minute virus of mouse (MVM) DNA, or poly(dA)·oligo(dT) as template–primer complexes.

The ribonucleotide analog, Bio-UTP, can substitute for UTP in reactions catalyzed by the RNA polymerases of *E. coli* and bacteriophage T7 (Fig. 2), although with a lower efficiency than that of any DNA polymerase/Bio-dUTP system. Furthermore, Bio-UTP is utilized poorly, if at all, by the eukaryotic RNA polymerases we have examined (HeLa cell RNA polymerase III, calf thymus RNA polymerase II, and mouse L-cell RNA polymerase II). Although the limited range of substrate function precludes the use of Bio-UTP in the direct enzymatic biotin labeling of eukaryotic transcripts *in vivo*, biotin-labeled RNA (Bio-RNA) probes can be prepared *in vitro* by using appropriate DNA templates and *E. coli* RNA polymerase or by 3'-end labeling methods using RNA ligase and biotin-labeled pUp (not shown).

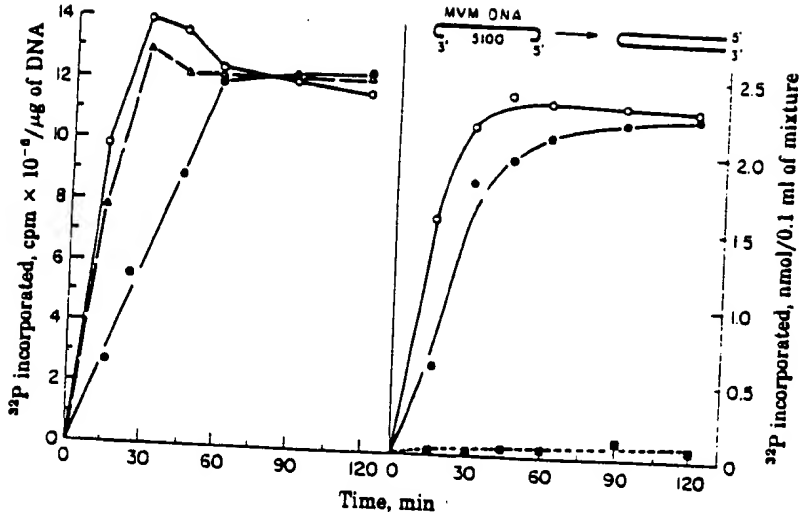

FIG. 1. Bio-dUTP is a substrate for *E. coli* DNA polymerase I. (*A*) λ Phage DNA was nick translated *in vitro* with DNA polymerase I holoenzyme as described (19). Reactions used [^{32}P]dATP (1.35 μM, 400 Ci/mmol) and either 20 μM TTP (○), 20 μM Bio-dUTP (●), or 10 μM TTP/10 μM Bio-dUTP (△). (*B*) Minute virus of mouse (MVM) DNA, a 5-kilobase single-stranded DNA with terminal hairpin duplexes (20), was converted to a double-stranded form by reaction with DNA polymerase I (Klenow fragment) as described (21). The three-nucleotide reaction (■; no TTP) contained dCTP, dGTP, and [^{32}P]dATP (50 μCi/μmol) at 0.1 mM. TTP (○) and Bio-dUTP (●) reactions were supplemented with the appropriate triphosphate at a final concentration of 0.1 mM.

The enzymatic polymerization of nucleotides containing biotin was not monitored directly because neither Bio-dUTP or Bio-UTP were radiolabeled. However, two lines of evidence show that the biotin-labeled nucleotides are incorporated. The first is that polynucleotides synthesized in the presence of biotin-labeled nucleotides are selectively retained when chromatographed over avidin-Sepharose affinity columns. For example, normal DNA, nick translated with TTP, dCTP, dGTP, and [^{32}P]dAMP, is quantitatively eluted from avidin-Sepharose by the addition of 0.5 M NaCl. In contrast, the majority of nick-translated biotin-labeled DNA (Bio-DNA) remains bound to the resin even after extensive washing with high salt, urea, guanidine·HCl, formamide, 2 mM biotin, or 50 mM NaOH (Table 1). The small fraction of radiolabel eluted by these washing conditions is not retained when it is applied to the resin a second time, suggesting that this radioactivity is associated with DNA fragments that are free of biotin substitution. Because the pBR322 DNA used in this experiment had ≈5% of its thymidine residues substituted by Bio-dUMP (based on picomoles of [^{32}P]dAMP incorporated in the nick-translation reaction), it is clear that only a few molecules of biotin per kilobase of DNA are necessary for irreversible binding to avidin-Sepharose. Indeed, when the "sticky" ends of Simian virus 40 DNA (linearized by treatment with *Eco*RI) are filled in by using Bio-dUTP and *E. coli* DNA polymerase Klenow fragment, the DNA is retained on avidin-Sepharose (unpublished data). Thus, four biotin molecules or fewer per five kilobases of DNA are sufficient for selective retention.

The second line of evidence for biotin substitution is that only polynucleotides synthesized in the presence of biotin-labeled nucleotides are immunoprecipitated when treated with purified antibiotin antibodies and then with formalin-fixed Staphylococcus (Table 2). Although the amount of biotin-labeled polymer found in the immune precipitate is dependent on the antibody concentration and time of incubation, under optimum conditions, >90% of the product can be immunoprecipitated, even when present in subnanogram quantities. Significantly, the results in Tables 1 and 2 show that the biotin molecule can be recognized by avidin and antibiotin antibodies when the DNA is still in a double-stranded form. Parallel experiments (not shown) indicate that biotin-labeled DNA·RNA hybrids and RNA duplexes behave similarly. These observations suggest that immunological and affinity methods could be used for probe detection (or isolation) following standard hybridization procedures.

To determine whether biotin-substituted polynucleotides were suitable for use as hybridization probes, the denaturation and renaturation characteristics of several biotin-labeled DNA and RNA polymers were examined. As shown in Table 3, the melting temperature of DNA duplexes decreases as the Bio-dUMP content of the polymer increases. A parallel analysis of RNA duplexes and DNA·RNA hybrids (not shown) indicates that they respond similarly. However, a pronounced decrease in melting temperature occurs only in heavily substituted polymers [e.g., poly(dA-dBio-U)] and even then the degree of coop-

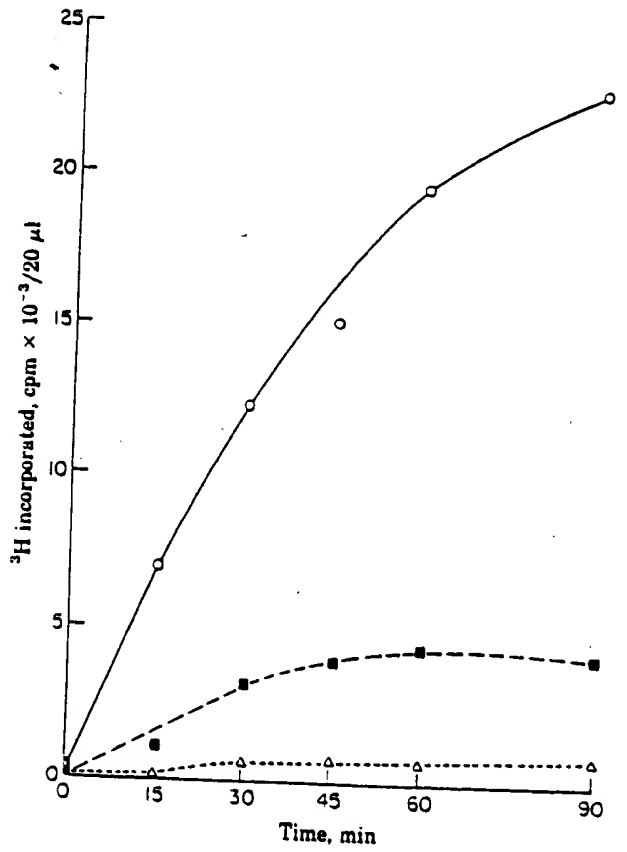

FIG. 2. Bio-UTP is a substrate for T7 RNA polymerase. Reaction mixtures (0.15 ml) were 40 mM Tris·HCl, pH 8.0/30 mM $MgCl_2$/10 mM 2-mercaptoethanol/0.4 mM ATP/0.4 mM GTP/0.4 mM [^{3}H]CTP (100 μCi/μmol)/0.4 mM UTP or Bio-UTP containing 7.5 μg of T7 DNA and 3.2 μg of T7 RNA polymerase. Aliquots (20 μl) were removed at the indicated times and acid precipitated onto glass-fiber filters. Curves: ○, UTP; ■, Bio-UTP; △, three-nucleotide reaction; no UTP or

Table 1. Selective retention of biotin-labeled DNA on avidin-Sepharose

	% DNA retained on resin	
	Bio-DNA (5%)*	Control
Load	100	100
Eluent		
0.5 M NaCl	100	0.1
1.0 M NaCl	99.7	<0.01
8 M Urea	100	<0.01
6 M Guanidine·HCl	95.2	<0.01
99% Formamide	94.7	<0.01
2 mM Biotin	97.6	<0.01
50 mM NaOH	89.5	<0.01

Avidin-Sepharose was prepared by coupling avidin to cyanogen bromide-activated Sepharose 4B essentially as described (22). Columns containing 0.2 ml of resin were equilibrated with 10 mM Tris·HCl/0.20 M NaCl, pH 7.5, and the DNA samples (3×10^5 cpm per load; 2×10^7 cpm/μg) were applied in 0.2 ml of the same buffer. The columns were washed with 1.0 ml of loading buffer and then treated with 2.0 ml of eluent. The % DNA retained on the resin was calculated by using the formula (cpm of DNA loaded − cpm eluted) ÷ cpm loaded, as determined by Cerenkov counting.

* pBR322 DNA labeled with [^{32}P]dAMP by nick translation in which 5% of the TMP residues have been replaced by Bio-dUMP.

erativity and the extent of hyperchromicity observed during denaturation are virtually identical to that of control polymers. Thus, pBR322 or λ DNAs that have been nick translated to introduce ≈20 biotin molecules per kilobase have melting temperatures similar to those of their biotin-free counterparts. Even MVM replicative form (RF) DNA in which every TMP residue in one strand (≈1250 in 5 kilobases) is replaced by Bio-dUMP has a melting temperature that is only 5°C less than that of the unsubstituted DNA. Of greater significance is the observation that lightly labeled DNA probes hybridize in solution at essentially the same rate as biotin-free probes (Fig. 3). Furthermore, ^{32}P-labeled biotin-substituted pBR322 DNA has the same degree of specificity and autoradiographic signal intensity as control biotin-free pBR322 DNA when used as a hybridization probe for detecting bacterial colonies that contain the plasmid (data not shown). These results indicate that a substantial number of biotin-labeled nucleotides can be introduced into a nucleic acid probe without significantly altering its hybridization characteristics.

Several additional properties of biotin-labeled polynucleotides are worth noting at this point. First, phenol extraction should be avoided whenever possible during purification of Bio-DNA or Bio-RNA because heavily substituted polymers are extracted into the phenol layer and even lightly or moderately substituted ones (e.g., nick-translated DNAs) can often be retained at the phenol/H_2O interface. Second, because the mass of Bio-dUMP is about twice that of TMP, extensive substitution can appreciably increase the overall mass of the polymer. For example, biotin-labeled MVM RF DNA (Fig. 1*B*) and restriction fragments derived from it migrate more slowly in agarose gels than their biotin-free counterparts (Fig. 4). Finally, incorporation of a biotin-labeled nucleotide into a restriction endonuclease recognition site may prevent enzymatic cleavage.

Table 2. Selective immunoprecipitation of Bio-DNA with antibiotin IgG and Staphylococcus

		Radioactivity, cpm	
DNA	Antibody	Precipitate	Supernatant
Control	—	70	4867
Control	Anti-Bio IgG	87	5197
Control	Nonimmune IgG	55	5107
Bio-DNA	—	53	3886
Bio-DNA	Anti-Bio IgG	3347	736
Bio-DNA	Nonimmune IgG	60	3900

Immunoprecipitation of DNA samples was done essentially as described (23). Biotin-labeled and control pBR322 DNAs labeled with [^{32}P]dAMP by nick translation (specific activity, 2×10^7 cpm/μg) were treated with 100 μl of formalin-fixed Staphylococcus (IgG Sorb, The Enzyme Center) in water for 10 min at room temperature. The supernatants from these reaction mixtures were incubated at 4°C for 1 hr without serum, with nonimmune rabbit serum, or with rabbit antibiotin affinity purified from serum provided by Fred Harmon. Immune complexes were precipitated by the addition of 50 μl of IgG Sorb. After 10 min at room temperature, the mixtures were centrifuged, and the pellets were washed three times with 30 mM Tris·HCl/150 mM NaCl/0.05% Nonidet P-40, pH 7.5, and analyzed by Cerenkov counting.

Table 3. Effect of biotin substitution on the thermal denaturation of DNA duplexes

DNA	Bio-dUMP content (% total nucleotides)	T_m, °C
Control pBR322	—	80
Biotin-labeled pBR322	2.0	79
Control MVM RF	—	69
Biotin-labeled MVM RF	12.5	64
poly(dA-dT)	—	62
poly(dA-dBio-U)	50.0	47

pBR322 DNAs were prepared by nick translation and thermally denatured in 10 mM Tris·HCl/50 mM NaCl/1.0 mM EDTA, pH 7.5. MVM RF DNAs were prepared as described in the legend to Fig. 1, and melting profiles were determined in 10 mM Tris·HCl/1.0 mM EDTA, pH 7.5. poly(dA-dT) and poly(dA-dBio-U) were prepared from *E. coli* DNA polymerase I reactions primed by poly(dA-dT) as described (24), and melting profiles were determined in 10 mM Tris·HCl/0.10 M NaCl/1.0 mM EDTA, pH 7.5.

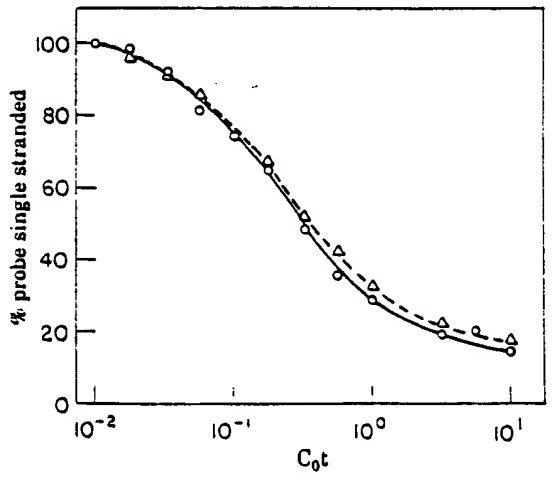

FIG. 3. Effect of biotin substitution on the reassociation rate of *E. coli* DNA. Sheared *E. coli* DNA was nick translated by using [α-^{32}P]dATP and either TTP or Bio-dUTP to a specific activity of 1.3×10^6 cpm/μg; the Bio-DNA probe had ≈5.5% of its TMP residues replaced by Bio-dUMP. The probes were heat denatured and hybridized at 37°C to a 220-fold excess of denatured nonradiolabeled *E. coli* DNA in 50% formamide/0.30 M NaCl/0.03 M sodium citrate, pH 7.0. Aliquots (10 μl) were removed at various times and diluted into 100 μl of 0.05 M sodium acetate, pH 5.0/0.05 M NaCl/1.0 mM $ZnCl_2$. Mung bean nuclease (2 units; P-L Biochemicals) was added, and the mixture was incubated at 42°C for 15 min. The amount of ^{32}P-labeled probe made resistant to the single-strand-specific nuclease was determined by acid precipitation onto glass-fiber filters. C_0t, initial concentration of DNA (moles of nucleotide/liter) × time (sec). ○, Control DNA; △, Bio-DNA probe.

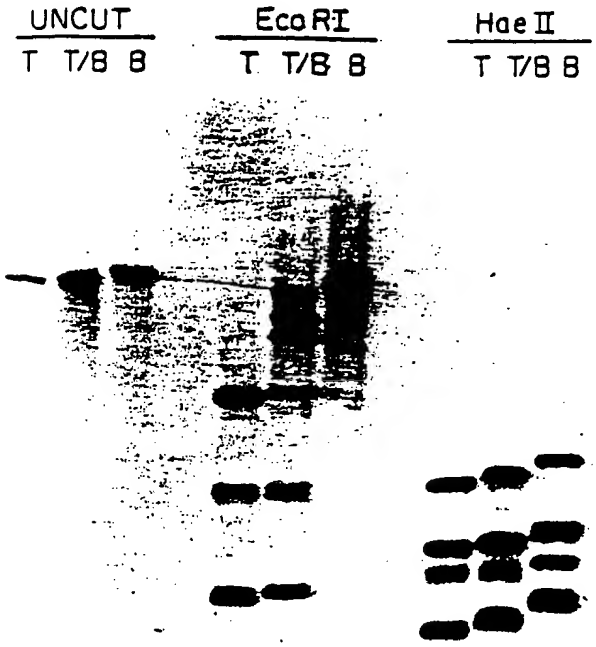

FIG. 4. Effect of biotin substitution on restriction endonuclease cleavage of DNA. ^{32}P-Labeled MVM RF DNAs prepared as described in the legend to Fig. 1*B* were cleaved with *Eco*RI and *Hae* II as described (20), and the samples were subjected to electrophoresis in a 1.4% agarose gel. Lanes: T, DNA gap filled with TTP as a substrate; T/B, DNA gap filled in the presence of equimolar concentrations of TTP and Bio-dUTP; B, DNA gap filled with Bio-dUTP. The faint bands in the *Hae* II lanes reflect a trace contamination of the enzyme with *Hae* III.

When Bio-dUMP is in the recognition site of *Eco*RI (G-A-A-T-T-C), the DNA is totally refractory to this enzyme although it remains sensitive to digestion by *Hae* II (PuG-C-G-CPy) (Fig. 4).

DISCUSSION

Our data demonstrate that Bio-dUTP and Bio-UTP are used as substrates by a number of nucleic acid polymerases, albeit at somewhat lower rates than the parent compounds, TTP and UTP. This provides a simple and rapid procedure for synthesizing chemically stable biotin-substituted polymers that hybridize specifically and efficiently to complementary sequences either in solution or bound to solid supports. Because polynucleotides containing a limited number of biotin molecules (50 or fewer per kb) hybridize with kinetics similar to those of unlabeled controls, standard hybridization protocols need be modified little if at all. The observation that Bio-DNA or Bio-RNA, and nonbiotinized sequences that hybridize to them, are selectively retained on avidin-Sepharose columns or immunoprecipitated by the addition of antibiotin antibodies and Staphylococcus is significant in several regards. First, these results suggest that biotin-labeled polymers can be used in conjunction with appropriate immunofluorescent, immunohistochemical, or affinity reagents for detecting or localizing specific sequences in chromosomes, cells, tissue sections, and blots. Our studies have led to the development of a rapid method of gene mapping by *in situ* hybridization that uses rabbit antibiotin antibody and fluorescein-labeled goat anti-rabbit IgG to identify the loci of hybridized Bio-DNA probes and a histochemical procedure for detecting biotin-labeled sequences on nitrocellulose filters that uses antibody–alkaline phosphatase conjugates (unpublished data). Second, the ability to synthesize immunogenic DNAs (and to a lesser extent RNAs) enzymatically, both in purified *in vitro* systems and in crude cell lysates, may allow the use of immunoprecipitation techniques. Finally, because the interaction between biotin-labeled polynucleotide probes and avidin-Sepharose is essentially irreversible, it should be possible to develop refined protocols for enriching (or deleting) specific gene sequences from complex mixtures in a fashion analogous to that reported by Manning *et al.* (25). Although further studies are obviously required, our results indicate that enzymatically biotin-labeled polynucleotides can function as nucleic acid affinity reagents.

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Nucleic Acid Probes

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Chapter 2

PREPARATION AND DETECTION OF NONRADIOACTIVE NUCLEIC ACID AND OLIGONUCLEOTIDE PROBES

J. L. McInnes and R. H. Symons

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Numerous methods are described in this chapter for the preparation by enzymatic and chemical techniques of nonradioactive nucleic acid and oligonucleotide probes. In many cases, the resulting probes have yet to be fully tested under hybridization conditions. In others, initial results look very promising since some nonradioactive probes can provide a sensitivity of detection of target sequences similar to that provided by ^{32}P-labeled probes.

II. ENZYMATIC PREPARATION TECHNIQUES

A. Preparation of Nonradioactive, Labeled DNA

1. Incorporation of Biotinylated Nucleotides into DNA

a. Nick Translation Reaction

A landmark in the development of methods for labeling nucleic acid probes by nonisotopic means was the publication of Langer et al.[1] describing the synthesis of biotinylated nucleotides and their enzymatic incorporation into DNA and RNA. The biotin was attached via a linker arm to the 5-position of the pyrimidine ring of dUTP (Figure 1) or UTP. Such nucleotides could be incorporated by the respective polymerases into DNA or RNA. The length of the linker arm between the biotin and the pyrimidine ring was found to be important in the subsequent detection of biotinylated DNA when used as a probe; linker arms of either 11 or 16 atoms were better than those with only 4 atoms (Figure 1) for both *in situ*[2] and dot-blot[3] hybridizations.

Biotinylation of double-stranded DNA is readily achieved in a standard nick translation reaction (see Chapter 1, Section II.A) catalyzed by *E. coli* DNA polymerase I. For example, bio-11-dUTP is incorporated in place of dTTP into the DNA to the same extent as dTTP but at a slightly slower rate.[1] Using standard nick translation procedures, substitution of between 20 to 70% of the available deoxythymidine residues with biotinylated dUTP can be achieved. Reassociation kinetics of denatured normal and biotinylated double-stranded DNAs were identical,[1] indicating that the biotinylated DNA exists as an unperturbed double-helix. Hence, established hybridization procedures can be used with biotinylated DNA probes prepared in this way.

In addition to the biotinylated dUTP analogues (Figure 1), a series of biotinylated dATP and dCTP analogues have been prepared which were incorporated into DNA probes by nick translation.[4] Bio-7-dATP and bio-7-dCTP (Figure 2) gave the highest incorporation in the series of analogues where N (the number of atoms between the carbonyl group of the biotin moiety and the amino group of adenine or cytosine) varied from 3 to 17. All the results obtained with DNA probes labeled with either bio-7-dATP or bio-7-dCTP were very similar to those obtained with bio-11-dUTP.[2,3] Hence, none of these biotinylated probes offers any advantages over the latter.

b. Replacement Synthesis Using T4 DNA Polymerase

As considered in Chapter 1 (Section II.D), T4 DNA polymerase can be used to generate high specific activity hybridization probes from double-stranded DNA. The same reaction can be used to incorporate bio-11-dUTP into DNA fragments with almost the same efficiency as the incorporation of dTTP.[5] The resulting biotinylated DNA probe was successfully used in colony hybridization.

c. Use of Other DNA Polymerases

Bio-dUTP is not a suitable substrate for the avian myeloblastosis virus (AMV) reverse transcriptase[1,6] in the preparation of long cDNA copies of RNA. However, the recently cloned form of the reverse transcriptase of Moloney murine leukemia virus[7,8] does incorporate bio-11-dUTP into full length cDNA, although somewhat less efficiently (35%) as compared

DNA PROBES

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Table 4.1 cont.

LABELING METHOD	DETECTION	REFERENCE	AFFILIATION	SENSITIVITY
CHEMICAL MODIFICATION				
oligonucleotides				
5'-amino	fluorescent primer	Smith *et al.* 1985	Cal Tech	NR
amino-cytosine	various groups	Ruth 1984	Molecular Biosystems	NR
amino-cytosine+ enzyme	direct	Jablonski *et al.* 1986	Molecular Biosystems	$3x10^6$ copies
5'-amino + enzyme	direct	Li *et al.* 1987	U. Adelaide	$3.5x10^6$ copies
		Sproat *et al.* 1987	EMBO	NR
SH-oligo + enzyme	direct	Chu and Orgel 1988	Salk Inst.	NR
Biotin-UMP	perox-Streptavidin	Cook *et al.* 1988	Enzo Biochem	$3x10^4$ copies
amino-cytosine+ enzyme	direct	Urdea *et al.* 1988	Chiron	$1.2x10^7$ copies

ENZYMATIC MODIFICATION

The first non-radioactive DNA probes, of a practical design, were described in the scientific literature by Langer *et al.* (1981). This early probe labeling scheme employed biotin-labeled deoxyribonucleotide triphosphates, incorporated into the probe DNA by enzymatic polymerization. The modified nucleotides, in turn, were developed as the result of years of experimentation with mercurated nucleotides and polynucleotides (Dale and Ward, 1975; Dale *et al.*, 1973; Dale *et al.*, 1975). The most widely used modified nucleotide is biotin-11-dUTP, as shown in Figure 4.1.

The molecule incorporates the following features: modification at the C-5 position where it will not interfere with hydrogen bonding, a double bond to minimize flexing of the linker arm and a linker arm long enough to ensure access of detection reagents to the biotin. This and other modified nucleotides can be incorporated into DNA by nick-translation (Leary *et al.*, 1983) or onto the ends of DNA by tailing (Riley *et al.*, 1986). After hybridization, these biotin-labeled probes are detected using avidin or streptavidin-enzyme conjugates. Streptavidin is superior to avidin for DNA detection because it exhibits far less nonspecific binding. Unlike avidin it contains no carbohydrate and has a neutral isoelectric point (Chaiet and Wolf, 1964). When combined with a precipitating substrate, the probe:immobilized target hybrid is visualized as a colored band or spot on nitrocellulose, (Leary *et al.*, 1983) or as cellular staining following *in situ* hybridization (Brigati *et al.*, 1983). These labeling and detection methods result in probes with a lower detection limit of 0.5-2 picograms, or about $5x10^4$ copies of target nucleic acid.

DNA Synthesis

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COVER: DNA polymerase bound to interruptions in a DNA molecule (from a photograph courtesy of Dr. Jack Griffith). See Fig. 4-6 for more detail.

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These discoveries and other important ones that followed led to the realization that DNA has two major and discrete functions. One is to carry the genetic information that brings about the specific phenotype of the cell; DNA is transcribed into RNA, and the RNA is then translated into the amino acid language of the proteins. The other major function of DNA is its own replication. For duplicating the genotype of the cell, DNA serves as a template for converting one chromosome into two identical chromosomes.

1960–present. The beginning of this age is not marked by a specific event. It is an age in which the generally held conceptions of both the structure and the dual functions of DNA have not been challenged but rather have been expanded. Without epochal discoveries, the justification for distinguishing this third age lies in a recent radical change in viewpoint toward DNA. Genetics and DNA have become a branch of chemistry. Despite its chemical complexity, DNA is being modified, dissected, analyzed, and synthesized in the test tube. There are insights into a metabolic dynamism of DNA that had not been anticipated. DNA suffers lesions and is repaired. DNA molecules exchange parts with one another. DNA molecules are specifically modified and degraded, twisted and relaxed, transcribed in reverse from RNA as well as directly into RNA. DNA functions not only in the nucleus but also in mitochondria and chloroplasts. There is now a stimulus to determine the total base sequence of DNA and to resynthesize it. There is also a confidence that the metabolic gyrations of DNA in the cell can be understood in as explicit detail as those of, say, glucose or glutamate.

2. The Primary Structure[7]

The two kinds of nucleic acid, the ribonucleic acids, RNA, and the deoxyribonucleic acids, DNA, are polymers of nucleotides.

A nucleotide (Fig. 1-1) has three components: (i) a purine or pyrimidine *base*, linked through one of its nitrogens by an N-glycosidic bond to (ii) a 5-carbon cyclic *sugar* (the combination of base and sugar is called a nucleoside) and (iii) a *phosphate*, esterified to carbon 5 of the sugar. Nucleotides occur also in activated di- and triphosphate forms, in which one or two phosphates are linked to the nucleotide by phosphoanhydride (pyrophosphate) bonds.

In each of the two main kinds of nucleic acids there are only four types of nucleotides. These are distinguished by their bases: adenine (A), guanine (G), uracil (U), and cytosine (C) in RNA, and adenine, guanine, thymine (T), and cytosine in DNA. The bases and their nucleoside and nucleotide forms are listed in Table 1-1. In base

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CHAPTER 7

THE NUCLEIC ACIDS

Structure and properties

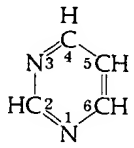
Nucleic acids may be divided into two classes, those containing ribose (ribonucleic acids, RNA) and those containing deoxyribose (deoxyribonucleic acids, DNA). Both are linear polymers of *nucleotides* (see below) that are formed by phosphodiester linkages between the 5′-phosphate of one nucleotide and the 3′-hydroxyl group of the sugar of the adjacent one.

COMPONENTS OF NUCLEIC ACIDS

Nucleotides consist of three components: a pyrimidine or purine base linked to a sugar, either ribose or deoxyribose, and phosphate esterified to the sugar at carbon 2, 3, or 5. Esterification at carbon 5 is most common.

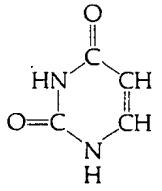
Pyrimidines

These are all derivatives of the parent heterocyclic compound *pyrimidine*. Its structure and the convention of numbering the positions in the ring are indicated below.

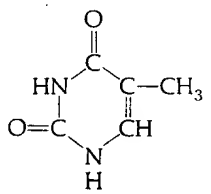


Pyrimidine

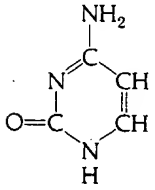
The principal pyrimidines found in RNA are uracil and cytosine; in DNA they are thymine and cytosine. Methylated and other pyrimidine derivatives are found in some nucleic acids (page 189).



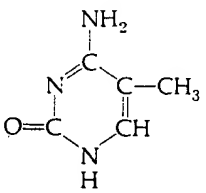
Uracil (2,4-dioxypyrimidine)



Thymine (5-methyl-2,4-dioxypyrimidine)



Cytosine (2-oxy-4-aminopyrimidine)



5-Methylcytosine (5-methyl-2-oxy-4-aminopyrimidine)

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18

STRUCTURE OF NUCLEIC ACIDS AND NUCLEOPROTEINS

Nucleic acids are long-chain polymers composed of nucleotides. The sequence of nucleotides is the repository of all genetic information carried by chromosomes. Despite this, not all nucleic acid is informational, nor is all the informational nucleic acid found in the chromosome. Examples of non-informational nucleic acid include ribosomal RNA and centromeric DNA, whose functions are primarily structural. Examples of informational nucleic acids not found in chromosomes include nucleic acids of mitochondria, chloroplasts, plasmids, and viruses. Most of the chapters in Part IV and Chapters 27 and 28 in Part V are devoted to explaining the ways in which nucleic acids are replicated and transmit their genetic information for use in the cell. In this chapter the focus is on the basic structural properties of nucleic acids in the free-solution state and as they exist in protein complexes in cells.

Electron micrograph of a human chromosome in late-prophase. (Magnification 21,000×.) The chromosome consists of two identical chromatids united at their centromeres. The chromatin consists primarily of a complex of DNA and histone (see text). It is still a mystery as to what forces cause the condensation of the nucleohistone into this highly condensed form. (Micrograph obtained from Gunter F. Bahr, M.D.)

NUCLEOTIDES, THE BUILDING BLOCKS OF NUCLEIC ACIDS

There are two chemically different types of nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Both DNA and RNA contain four different nucleotides. Each *nucleotide* contains a nitrogenous base known as a *purine* or a *pyrimidine*; a sugar, *ribose* in RNA; *deoxyribose* in DNA; and a phosphoryl group. The nucleotide may be converted to a *nucleoside* by removal of the phosphate. The primary structure of the four commonly occurring deoxyribonucleotides found in DNA are shown in Figure 18-1.

R. Rieger · A. Michaelis · M. M. Green

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DNA helix groove faces outwards from the protein core. The particle mass (~206 kDA) of the n. core is equally distributed between 146 (±2) bp of DNA and the histone octamer. The fifth histone (H1) interacts with an additional 20 bp of DNA to complete two turns of DNA around the histone core.

The n. cores are then linked by DNA (linker DNA) less intimately associated with histones to form structures that appear as "beads on a string". The amount of linker DNA varies from organism to organism, cell type to cell type within an organism, and even within chromatin of a single nucleus. This results in an overall n. size of around 160 bp to about 240 bp (200 bp in most vertebrates).

The structural and mechanical properties of DNA change according to its base sequence, and therefore the ability of a DNA molecule to bend around the histone octamer is thought to be a major determinant of nucleosome positioning.

The principal function of nucleosomes is to compact DNA into the 30-nm filament; transcriptionally active DNA sequences possess special features that permit → RNA polymerase access and passage (→ nuclease hypersensitive site). Three levels of chemical heterogeneity may contribute to this transcriptionally active state: (1) Different primary structures of the core histones create the potential to generate (in mammals) 180 compositionally unique histone octamers; (2) the core histone subtypes are post-translationally differentially modified (by acetylation, methylation, phosphorylation, poly(ADP-ribosyl)ation, and ubiquitation); (3) histone H1 subtypes, → high mobility group proteins, and other accessory proteins interact with specific binding sites on nucleosomes.

Essentially all the nascent core histones are conservatively deposited and segregated in nucleosomes. They bind preferentially to newly synthesized DNA; parental histones segregate to one arm of the DNA replication fork (the leading side for → DNA replication). The conservative assembly and segregation of n. cores is a means whereby information controlling gene structure and expression can be stably incorporated into chromatin.

There are two extreme models to account for the folding of DNA in the n.: (1) A smooth, continuous bending of the DNA (2) localized distorsions at regular points ("kinks") with straight regions of multiples of 10 bp.

nucleosome core particle — any of the particles derived from → chromatin by digestion of linker DNA sequences and removal of histone H1. The n.c.p. consists of 146 (±2) bp of DNA wrapped around the outside of an octamer of histones (→ nucleosome). It is a flat cylinder (5.5 nm high and 11 nm in diameter). A special type of n.c.p. is called → chromatosome. At least three types of interactions are involved in n.c. assembly: (1) Stereospecific bonding between histones to form octamers; (2) electrostatic repulsion between the charged histones; (3) electrostatic attraction of the histones for DNA (→ nucleoplasmin).

nucleosome filament — a linear array of connected → nucleosomes which abut each other (diameter of the n.f. 10 nm). The n.f. is the → chromatin fiber and can be coiled into a solenoid, thereby forming a 20—30 nm diameter filament or "thick fiber" which may be further folded into a radial loop with the loop bases organized into the central portion of the → chromatid.

nucleosome linker — → linker DNA.

nucleosome phasing (McGhee and Felsenfeld 1980) — the preferred positioning of the → nucleosome core histone octamer with respect to the sequence of the DNA wrapped around it. Three different situations of n.p. are known: (1) The distance of regularly spaced nucleosomes may be related to the repeat length of a reiterated DNA base sequence; (2) not regularly spaced nucleosomes may be specifically positioned; (3) regularly spaced nucleosomes may be associated in one or several distinct modes with a given DNA sequence.

nucleosome spacing — the disposition of → nucleosome cores one to another, i.e., the average length of → linker DNA which may vary from 20 to 100 bp depending on the organism or tissue under study.

nucleotide (Levene 1909) — any of the monomeric units which are the building blocks of the

polynucleotides referred to as → nucleic acids (Table 10). A n. is a phosphate ester of the N-glycoside of a nitrogenous base and consists of a purine or pyrimidine base, a pentose (D-ribose in → ribonucleic acid, 2′-deoxy-D-ribose in → deoxyribonucleic acid) and a phosphate (PO_4) group. The combination of nitrogenous base and sugar is called a ribonucleoside (when the pentose is D-ribose) and a deoxyribonucleoside (when the pentose is 2′-deoxy-D-ribose). The nucleotides are formed by the addition of a phosphate group to the nucleosides and are either ribonucleotides (in RNA) or deoxyribonucleotides (in DNA).

Table 10. The main components of DNA and RNA

Nucleic acid	Main purine (PU) and pyrimidine bases (PY)	Pentose	Nucleoside	Nucleotide
		2′-deoxy-D-ribose	Deoxyriboside	Deoxyribotide
DNA	Cytosine (PY)		Deoxycytidine	Deoxycytidylic acid
	Thymine (PY)		Deoxythymidine	Deoxythymidylic acid
	Adenine (PU)		Deoxyadenosine	Deoxyadenylic acid
	Guanine (PU)		Deoxyguanosine	Deoxyguanylic acid
		D-ribose	Riboside	Ribotide
RNA	Cytosine (PY)		Cytidine	5′Cytidylic acid
	Uracil (PY)		Uridine	5′Uridylic acid
	Adenine (PU)		Adenosine	5′Adenylic acid
	Guanine (PU)		Guanosine	5′Guanylic acid

nucleotide-excision repair — a type of → DNA repair which removes damaged bases from DNA as an oligonucleotide (→ excision repair; base-excision repair). The resulting gap is patched by → repair synthesis. Mono- and diadducts are removed by n.-e.r. (interstrand croslinks are repaired by concerted action of n.-e.r. and → recombinational repair pathways).

nucleotide replacement site — any position in a → codon where, due to → gene mutation, a base has been substituted.

nucleotide sequence — a specifically ordered row of → nucleotides in the DNA or RNA of organisms. A n.s. which occurs many times in the DNA of one cell is called a repeated n.s. (→ repetitious DNA). A particular n.s. which occurs only once in a genome is called a unique n.s. (→ unique DNA).

nucleotide sequencing — → DNA sequencing.

nucleotide substitution — the presence of different nucleotides in equivalent positions of homologous DNA or RNA sequences.

nucleotide synthesis — in virtually all cells, synthesis of → nucleotides by two fundamentally different kinds of pathways: (1) The **de novo pathway** in which ribose phosphate, certain amino acids, CO_2 and NH_3 are combined in successive reactions to form the nucleotides (free bases are not used as intermediates); (2) the **salvage pathway**, in which free bases or nucleosides, produced by breakdown of nucleic acids, are converted back to the nucleotides.

nucleotidyl cap — → messenger RNA capping.

nucleotype (Bennet 1972) — the gross physical characteristics of the → nucleus, its mass, and particularly the amount of DNA contained in its constituent chromosomes.

nucleus (Brown 1831) — a membrane-enclosed cell organelle which represents one of the two main compartments of the eucell and contains the bulk of its → genetic information (nuclear DNA) in the form of → chromatin. Replication of chromosomal DNA, → genetic transcription of DNA and processing of DNA transcripts (→ heterogeneous nuclear RNA; messenger RNA, ribosomal RNA, transfer RNA) occur in the n.

W. Guschlbauer

Nucleic Acid Structure

AN INTRODUCTION

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To Marie-Pierre

To Florian, Theresia, and Willi

To "explain" an event means to trace or "reduce" its regularities to more general laws of nature. If the event occurs within a system consisting of several different parts, a knowledge of their form and function is, in any science, indispensable to successful reduction. While physicists are interested in structure only as a means to this end, biologists regard a knowledge of structure as an end in itself. The current belief that only quantitative procedures are scientific and that the description of structure is superfluous is a deplorable fallacy, dictated by the "technomorphic" thought-habits acquired by our culture when dealing preponderantly with inorganic matter.

Konrad Lorenz (1971)

to act as competitive inhibitors), or, if they are incorporated into the enzymatic product, they will be fixed in a rather strict conformation and thus limit function (e.g., in DNA).

3.1.4 PRIMARY STRUCTURE OF RNA AND DNA

Nucleosides are linked through a phosphodiester bond via the 3′ and 5′ oxygens to give the primary structure of the nucleic acids, which is ubiquitous and, without exception, the same (Fig. 3.7). The differences between DNA and RNA will be evoked in connection with the corresponding model compounds (Chapter 6, Section 6.2.3). It is essentially due to differences in the sugar pucker: DNA shows the 3′-*exo* conformation, RNA the 3′-*endo* conformation.

3.2 Chemical Reactions of Nucleic Acids and Their Constituents (6)

3.2.1 REACTIONS OF THE SUGAR MOIETY

Periodate reaction. One of the most widely employed reactions in RNA chemistry is the periodate oxidation of the *cis*-glycol of ribose (2′ and 3′-OH). No reaction occurs with deoxyribose or arabinose. This reaction yields the terminal nucleoside as 2′,3′-dialdehyde and is useful in identifying terminal nucleosides in RNA and/or coupling them with other reagents.

Color reactions. Two very old methods, which are still widely used, depend on the hydrolysis of the glycosidic linkage of purine nucleosides by hot concentrated acids, with the subsequent transformation of the sugar into furfural derivatives. These derivatives, in turn, give specific color reactions with such aldehyde specific reagents as orcinol, which reacts with furfural (from ribose) and ferric chloride to give a green color; the intensity of this color is proportional to the ribose concentration. A blue color is obtained with deoxyribose in concentrated sulfuric acid with diphenylamine (7).

3.2.2 CHEMICAL REACTIONS OF BASES

A very large number of reactions, described in the literature, imply the modification of the nucleic acid bases. A few of the most useful and widely used ones are discussed.

Nitrous acid. Sodium nitrite at low pH reacts with aromatic amines to form phenols. This reaction has been widely used to induce chemical mutations (see Chapter 7). Since the phenolic forms of the bases are not stable, the corresponding keto compounds are obtained:

Ade→Hyp, Gua→Xan, Cyt→Ura.

The reaction rate decreases in the order Cyt > Ade > Gua. Furthermore, exposed amino groups are more susceptible to attack than those involved in a structure or H-bonds.

Sodium nitrite therefore acts more efficiently on single-stranded nucleic acids than on double-stranded ones.

Dilute nitrous acid attacks Ura to yield 5-nitrouracil, which has a characteristic UV spectrum (peak at 350 nm).

Formaldehyde. Formaldehyde (like many other aldehydes or ketones) reacts with the amino groups of free bases or single-stranded nucleic acids. Since it can react with accessible amino groups, formaldehyde is used to measure the kinetics of nucleic acid unwinding (8). The chemistry is not well understood (possibly a Schiff's base as an intermediate). There is a detailed review by Feldman (9) on the subject.

Hydroxylamine. Hydroxylamine is a strong mutagen and reacts specifically with Cyt and hom^{5}Cyt (5-hydroxy-methylcytosine); but it also reacts less strongly with Thy and Ura. The former reaction is probably a two-step one, the first step being the addition of NH_2OH on the C^5-C^6 double bond, then the elimination of two moles of ammonia, and water, and finally, the formation of Ura.

Halogenation. Like many compounds, nucleic acid bases are attacked by halogens, the pyrimidines at position 5, the purines at position 8. Halogenobases have found wide application in chemotherapy (see Chapter 7), br^{5}dUrd is a powerful dThd analogue; br^{8}Ado and br^{8}Guo have been used in RNA polymerase studies.

Alkylation. Dimethylsulfate (or other alkylating agents) act on nucleosides and nucleic acids to yield alkylated bases, which are frequently powerful mutagens or carcinogens. The alkylation site is generally that of protonation or amino groups, i.e., N^7 (and N^1) in Gua and Ino, N^1 and N^6 in Ade, N^3 and N^4 in Cyt. Since they frequently substitute at the site where hydrogen bonds are formed, aberrant pairings are expected. Bi-functional alkylating agents, like nitrogen mustards ($Cl\text{-}CH_2\text{-}CH_2\text{-}NR\text{-}CH_2\text{-}CH_2\text{-}Cl$), or some antibiotics, like mitomycin, can induce cross-links between two strands of DNA by reacting with two Gua on opposite strands.

Other reactions. The reaction with P_2S_5 permits the obtention of sulfur-substituted bases, which are useful intermediates. These mercaptols (with a sulfur rather than a keto group substituent) can be treated with alcoholic ammonia to yield amines.

The literature on the chemistry of nucleosides and nucleotides is enormous. Several classics exist on this subject (e.g., Ref. 10).

Photochemistry (11). Ultraviolet irradiation of pyrimidines in aqueous solution adds a water molecule to the C^5-C^6 double bond. This reaction is reversible upon heating. If the solution is frozen, or the bases are otherwise in a rigid

structure (crystals, polymers, DNA), dimers are formed between the two pyrimidines, which are thus connected through the C^5 and C^6 bonds in a cyclobutane structure, although mixed Cyt-Thy (or Cyt-Ura) dimers have also been observed. In DNA, UV irradiation forms dimers, which have a mutagenic effect. In the cell, there are specific enzymes that excise such Thy dimers and repair the break (see Chapter 4, Section 4.3.1).

3.3 Isolation of Nucleic Acids (12–14)

In order to study the nucleic acids by physicochemical or enzymological methods, they must be isolated and purified from other cell constituents. As their name indicates, they are acidic in character (one negative charge per nucleotide residue) and are neutralized by basic proteins (protamines, histones), polyamines (spermine, spermidine, etc.), or metallic cations (alkalis, earth alkalis). Nucleic acids are irreversibly denatured, if all their basic components are removed. For this reason, extractions are usually carried out in salt solutions buffered at pH 7.

On the other hand, DNA, being a long thin thread (20 A in diameter, several microns to millimeters or more in length), is very fragile and easily damaged by shear and mechanical forces and enzymatic activity. Great care has, therefore, to be taken to avoid breakage, if one hopes to isolate a native intact DNA. Pipetting may cause DNA breakage. Upon isolation, the DNA molecule is tested for purity and physical integrity (e.g., by sucrose density gradient centrifugation). Polysaccharides are a big problem, since they are difficult to eliminate, especially when the DNA is isolated from animal or plant tissue. The isolation procedure has to be adapted to a given purpose. Thus, it is generally not possible to isolate DNA and RNA in native form in a single procedure.

In a general procedure, after the cell wall is broken by mechanical or enzymatic methods (lysozyme), the resulting cell sap is treated with a protein-denaturing agent, such as phenol, or a detergent (dodecyl sulfate, lauryl sulfate), which precipitates proteins. Several extractions are frequently necessary. The final nucleic acid solution is treated with ethanol, to precipitate nucleic acids, or dialyzed against a suitable buffer solution. A review by Kirby (14) discusses the various isolation procedures used and their advantages and inconveniences.

3.4 egradation and etermination of Nucleic Acids

3.4.1 CHEMICAL DEGRADATION

Hydrazinolysis yields apyrimidinic (i.e., DNA in which all the pyrimidines have been split at the glycosidic linkage).

Dilute acid selectively cleaves the glycosidic linkage of purines in DNA, yielding apurinic acid. It does not act on RNA, except when hot and very concentrated (see Section 3.2.1).

RNA is hydrolyzed quantitatively by 0.3 M KOH at 37°C for 24 hours, while DNA is denatured, but not hydrolyzed. This difference is the basis of the separation method of Schmidt and Thannhauser (15). The alkaline hydrolysate is treated with cold perchloric or trichloroacetic acid. The ribonucleotides remain in solution, while the denatured DNA is precipitated (with the remaining proteins) and separated by centrifugation and washing. The supernatant, containing the ribo-3′-phosphates, can now be analyzed by color reactions, chromatography, etc., while the precipitate is hydrolyzed further by acid, to be analyzed as above. This method is rather widely used for measuring the radioactivity of DNA, RNA, or proteins in *in vivo* labeling experiments using radioactive precursors. These methods give—at best—the base composition of the material under investigation, but no information about the sequence is obtained. Furthermore, due to the rather harsh treatment, losses are inevitable, and there are many sources of error.

3.4.2 ENZYMATIC DEGRADATION

There exists a number of nucleases, some of which are highly specific (Fig. 3.8). One distinguishes between endonucleases, which attack within the nucleic acid chain, and exonucleases, which attack sequentially at a given end of the polynucleotide chain.

Pancreatic ribonuclease. Ribonuclease (MW 13,600), with known amino-acid sequence and tertiary structure, is quite heat stable and frequently a great nuisance, because of its ubiquitous presence and its stability. It attacks only single-stranded RNA and cleaves at the 5′-side of pyrimidine nucleotides (see Fig. 3.8). The reaction, therefore, yields oligonucleotides with -Yp, i.e., with the 3′-phosphate group on the terminal pyrimidine nucleoside. Its reaction mechanism is well known and closely follows that of alkaline hydrolysis, i.e., the intermediate is a pyrimidine nucleoside-2′,3′-cyclic phosphate.

Takadiesterases T_1 and T_2. The first of these two endonucleases splits RNA after Guo, in a way analogous to that of RNAase, and yields Guo-3′-P, while takadiesterase T_2 splits after Ado and yields Ado-3′-P.

Snake venom phosphodiesterase and polynucleotide phosphorylase. Snake venom diesterase is an exonuclease that hydrolyzes both DNA and RNA; it can be isolated from the venom of many poisonous snakes. It attacks sequentially from the 3′-OH end and yields 5′-phosphates. Polynucleotide phosphorylase (PNPase) has a similar mode of action. As the name indicates, it is a phosphorylase and not a

BASIC PRINCIPLES IN NUCLEIC ACID CHEMISTRY

VOLUME II

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1

CHEMICAL REACTIONS OF POLYNUCLEOTIDES AND NUCLEIC ACIDS

D. M. BROWN

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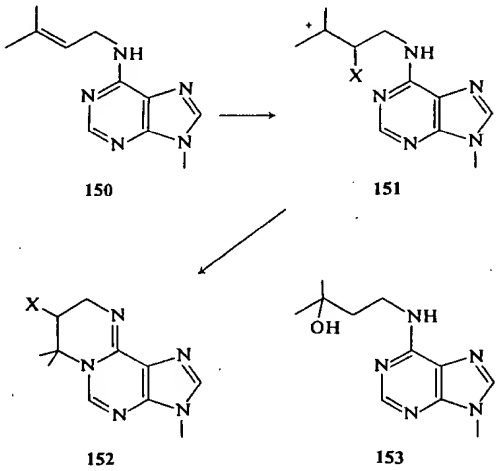


150 151 152 153

4-Thiouridine is oxidized rapidly by iodine to the 4,4-disulfide [237]. This reaction, too, occurs in *E. coli* $tRNA^{Tyr}$ in which two thiouridine residues are in contiguous positions and is not a result of intermolecular coupling [238–240]. Even in tRNA's having only one 4-thiouridine residue a reaction occurs, which (like disulfide formation) is reversible by thiosulfate. The iodine reaction with 2-methylthio-N_6-isopentenyladenosine may be reversible by thiosulfate [236].

We may conclude, with respect to tRNA, that iodine is among the most specific of the base-modifying reagents.

IV. Reactions Affecting the Internucleotide Linkage

A. Introduction

In this section we will discuss reactions which lead, directly or indirectly, to the cleavage of the internucleotide linkage. The cleavage reactions themselves fall into two classes: those that proceed by hydrolysis (P—O cleavage) and those that proceed by an elimination (C—O cleavage). In the former, the very much greater susceptibility of the polyribonucleotides as compared with their 2′-deoxy analogs is well known. In the latter, the presence of a carbonyl (or related) function β to the phosphate residue is essential to activate the elimination process. Thus, for example, glycerol 1-phosphate is highly stable to base, whereas glyceraldehyde 3-phosphate is extremely labile even at pH values near neutrality. In terms of nucleotides, elimination reactions

are important in situations where the glycosidic linkage has been broken, that is, when the heterocyclic aglycone residue has been lost, or when a carbonyl function has been introduced into the sugar residue by oxidation.

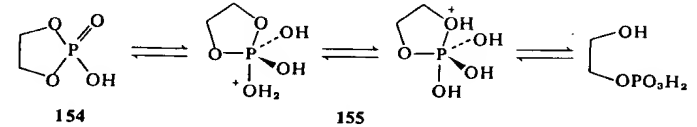
$$^{2-}O_3POCH_2CH(OH)CHO \longrightarrow PO_4^{3-} + CH_3COCHO \xrightarrow{OH^-} CH_3CH(OH)CO_2^-$$

It is important in nucleotide sequence work, where enzymic methods are not applicable (or as an adjunct to these) that some means of increasing the specificity of cleavage of a given internucleotide link should be available. Elimination, in contrast to hydrolysis, can provide the necessary rate difference. In principle, too, changing the degree of esterification of the internucleotide phosphoryl residue can achieve this and in this connection we will discuss phosphate alkylation briefly.

A number of hybrid molecules containing ribo- and deoxyribonucleotides are known, for instance, the RNA-linked DNA replicative fragments from *E. coli* and other sources. Their hydrolytic chemistry is exactly as expected from the known chemistry of RNA and DNA [240a].

B. Phosphate Diester Hydrolysis

Simple dialkyl phosphates, considered as analogs of the internucleotide linkage in DNA, are extremely stable to base and, moreover, hydrolysis, when it does occur, probably does so by alkyl-oxygen cleavage. Acid-catalyzed hydrolysis is also slow. Hydrolysis rates are increased dramatically by neighboring-group participation, a matter which has been studied much in recent years though mainly in compounds other than nucleotides [241]. The 5-membered cyclic phosphates are exceptional when compared with their acyclic analogs. Thus ethylene phosphate (**154**) is hydrolyzed by acid and by base in the region of 10^7–10^8 times faster than dimethyl phosphate [242]; ribonucleoside 2′,3′-phosphates equally are very rapidly hydrolyzed [243]. Under both conditions P—O cleavage alone occurs [244]. Studies of this and related phenomena in recent years have led to a wider understanding of phosphate ester chemistry [245]. Briefly, it appears that ring strain can account for much (but not all) of the observed rate increase. Mechanistically, the strain can be released by going to a trigonal bipyramidal pentacoordinate intermediate (**155**) by addition of water, the ring including one apical and one basal bond, and hence a 90° O—P—O bond angle. The intermediate can then break down by ring cleavage, or, competitively, by loss of an exocyclic substituent, manifesting itself in rapid ^{18}O-exchange in $H_2{}^{18}O$-enriched solvent. The concept of pseudorotation [246] is important in this context; that is the fast exchange of the apical (a) and basal (b) ligands about phosphorus (**156**) as a result of a deformation process (**157**). Groups may enter or leave only at apical positions [245].



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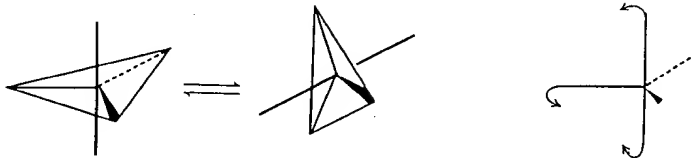
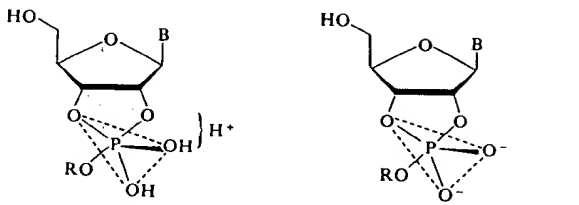


156 157

These considerations have a bearing on the hydrolysis of ribonucleotide esters (or polyribonucleotides) in which the neighboring 2′-hydroxyl group is involved. Under acid catalysis, a pentacoordinate intermediate (**158**) is assumed to be formed which can undergo pseudorotation allowing the C_2·O—P, the C_3·O—P and the exocyclic RO—P bonds to become apical, whence bond fission leads to starting ester, rearranged ester, and cyclic phosphate, as observed [247]. The possibility of alkylphosphoryl group migration is important in synthetic studies since such rearrangement may occur during removal of acid-labile protecting groups. Choice of suitable protecting groups has, however, essentially obviated this danger. Fortunately there is no evidence that migration occurs in polynucleotides under conditions in which hydrolysis is inappreciable.

In polynucleotide structural studies base-catalyzed hydrolysis is more generally applicable because, under these conditions glycosidic linkages are stable. It is not clear whether pentacoordinate intermediates (**159**) are important in the hydrolysis of nucleoside 2′- or 3′-phosphate esters (e.g., **160**). Evidence that no migration of the alkyl phosphoryl group (2′ ⇌ 3′) occurs has been given so that an "in-line" displacement mechanism probably pertains, through the $S_N2(P)$ transition state (**161**) [247,248]. Alternatively the pentacoordinate intermediate (**159**) is formed but cannot pseudorotate since this would put the electropositive —O^- ligands into an apical position, energetically an unfavorable state [249]. It is interesting that ribonuclease catalysis of both dinucleoside phosphate and cyclic phosphate hydrolysis proceed exclusively by "in-line" mechanisms [250,251]. Another interesting sidelight is that a 3′,5′-linked polynucleotide in a helical state is in a conformation which precludes hydrolysis by an "in-line" mechanism (see also Section V,C, 2). This is not so for a 2′,5′-linked polymer. It is therefore conjectured that the greater stability achieved, as a result, may account for the prebiotic establishment of the now universal 3′,5′-linkage [252]. By the principle of



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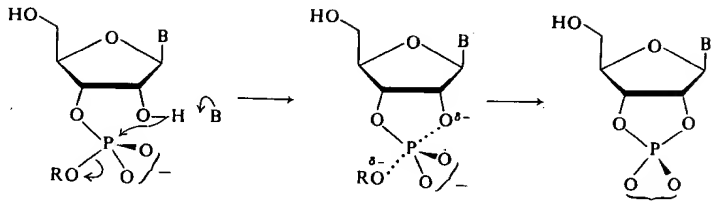

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microscopic reversibility it accounts, too, for the observation [253] that chemical generation of oligonucleotides from nucleoside 2′,3′-phosphates oriented on complementary polynucleotides leads almost exclusively to products with the 2′,5′-linkage. Polymerization using nucleoside 5′-polyphosphates chemically or, presumably, enzymically does not, of course, suffer from this mechanistic restriction.

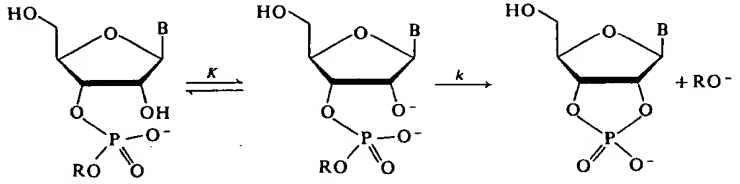
Macromolecular DNA in solution undergoes a slow reduction in molecular weight with time [254]. We shall discuss the question in more detail later, but meanwhile one conceivable explanation is that distortion of an internucleotide linkage to a state analogous to that in 5-membered cyclic phosphates increases the rate of hydrolysis sufficiently at that position to account for the observed rate of molecular weight decrease. Single-strand scission of *E. coli* (or λ duplex) DNA in 0.3 *N* NaOH occurs at a rate of one break/10^6 daltons at 25°C [254a]. Double-strand cleavage results from the application of hydrodynamic stress [255,256]. The mechanism of the shearing process is unknown and no work has been reported on the nature of the end groups in such sheared molecules. The process may be hydrolytic as above, but alternatively sufficient strain could be localized in a bond to allow homolytic (radical) cleavage and calculation suggests that this is so [256].

C. Polyribonucleotide Hydrolysis

In the previous section the mechanism of base-catalyzed hydrolysis of ribonucleotide esters was discussed briefly. It is important to consider the

factors which may apply in determining the rate of the process. The *cis*-stereochemistry of the neighboring 2′-hydroxyl group in the furanose system is probably optimal. A *trans*-2′-OH group as in *arabino*-nucleotide esters is ineffective as a neighboring group [257].

The acidity of the neighboring function in **162** determines the concentration of **163** and hence the rate of hydrolysis under a given set of conditions. In other series, rate measurements are consistent with the view that $k_{obs} = Kk$ [258]. The factors influencing the rate are difficult to assess because the acidity of the neighboring OH group and the nucleophilicity of the derived alkoxide anion are not simply related. It is also observed that the rate increases with the increased stability of the leaving group RO^- and that a plot of log k_{obs} vs pK_a of ROH is linear over a wide range of pK_a [248,158]. Consistently a very low rate of hydrolysis of **162** (R = Pr^i) is found compared with **162** (R = Me or nucleoside-5′) [259]. Metal ion catalysis is another important factor which is little understood in detail (see below).



162 **163**

All of these factors must play a part in determining the rate of hydrolysis of the internucleotide linkages in the 16 possible dinucleoside phosphates (and in the corresponding linkages in polynucleotides). One further factor may be the stereochemical restraint imposed on polynucleotides by stacking interactions—these interactions being maximal in aqueous solution [260]. The nature of the base and its state of ionization will be important in this connection. It may be assumed that the nucleophilic activity of the neighboring hydroxyl group and the leaving capacity of the 5′-linked nucleoside residue should, as a first approximation, be taken as constant and variations in rate of hydrolysis would depend on stacking interactions, the latter possibly being disrupted by ionization, particularly of guanine and uracil residues [2].

The evidence available (Table II) shows that dinucleotides of the form PupNp undergo hydrolysis more slowly than those of the form PypNp and that the 3′-linked component has less effect on the rate than the 5′-linked one. There is, thus, about one order of magnitude difference between the fastest and slowest dinucleotide, the latter being ApAp [261,262]. Dinucleoside phosphate [263] and RNA [264–266] hydrolyses give comparable results. These observations are in general agreement with the views expressed above.

TABLE II

RELATIVE RATES FOR THE HYDROLYSIS OF SOME DINUCLEOTIDES IN 0.86 *M* KOH AT 26°C[a]

UpUp	1.0	ApUp	0.25
CpC3p	0.62	ApGp	0.21
CpC2P	0.38	GpAp	0.18
GpCp	0.25	ApAp	0.10

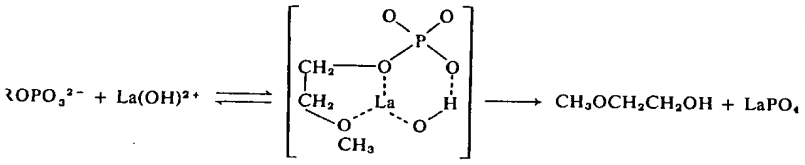
[a] From Lane and Butler [261].

It is worth recalling here that alkaline hydrolysis of RNA can be accompanied by modification of some bases. For example, 1-methyladenine rearranges to N_6-methyladenine [87], 3-methyluracil is degraded [267], 3-methylcytosine [268], and, more slowly, cytosine [269] are deaminated, while 5,6-dihydrouracil [270], and 7-methylpurines [271] are ring-opened. 2′-*O*-Methylnucleosides appear in hydrolysates as alkali-stable dinucleotides [272,273].

In general it seems that the difference in rate of hydrolysis between one internucleotide linkage and another by acid- or base-catalyzed hydrolysis is not sufficient to have any useful application in structural work. It is also true that the pH conditions, being at the extremes of the scale, are not such as to allow the retention of secondary structure. Catalyzed hydrolysis which occurs around neutrality is much more likely to be useful and this can be effected by means of heavy metal ions.

Metal ions may act as electrophilic catalysts in the hydrolysis of phosphate esters. In no case is the catalysis understood in detail, and in many cases it is not possible to obtain rate data as the reactions do not always occur in homogeneous solution. Nevertheless, the rate enhancements can be very large. Phosphate monoesters have been studied intensively. La^{3+} and Ce^{3+} are very effective [274,275]. It is often noted, as it is with diester hydrolyses, that the reactions are sharply pH-dependent and that the optimum pH may vary with the concentration both of the metal ion and of the substrate. A neighboring oxygen function has an important effect on the rate and may be involved, in some cases, in metal binding. For example, the lanthanum ion catalyzed hydrolysis of 2-methoxyethyl phosphate at pH 8.5 has been thought of as proceeding through a complex such as **164** [276].

Turning to polynucleotides, it appears that in general, the polyribonucleotides are much more susceptible to metal ion-catalyzed hydrolysis than polydeoxyribonucleotides [274]. Products range from oligonucleotides through nucleoside-2′,3′ phosphates and nucleotide-2′ (and -3′) phosphates to nucleosides [277–281]. Many metal ions, including Ca^{2+}, Zn^{2+} and Ba^{2+},



164

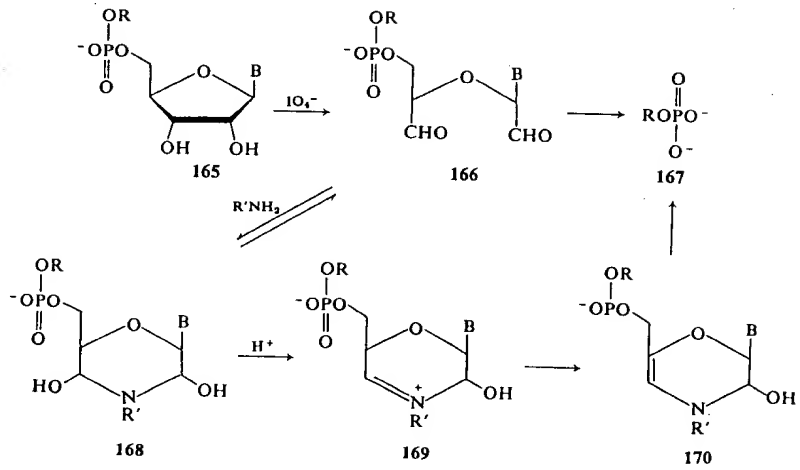
act catalytically, the effect being noted *inter alia* by loss in biological activity (e.g., of TMV–RNA) or molecular weight diminution if not by product detection [282]. The Pb^{2+} ion has the largest catalytic activity so far observed [274,280,281,283]. Thus at 1.1 m*M* concentration and pH 7.5 rapid depolymerization of polyribonucleotides occurs. With homopolynucleotides the observed rate order is poly A ≃ poly U ≫ poly I > poly C. Secondary structure protects against hydrolysis so that poly U·poly A is hydrolyzed at one-third the rate of either component and poly I·poly C is stable. Transfer RNA is in part hydrolyzed, the rate being reduced by added Mg^{2+}, while, surprisingly, CpC is unaffected. It is evident that behind these observations must lie much interesting and possibly useful chemistry [280,283].

D. Phosphate Elimination Reactions

In this section we deal with phosphate elimination reactions. Their importance to nucleic acid chemistry is due to the fact that conditions may be found which lead to internucleotide bond cleavage under conditions, both acidic and basic, much milder than those that pertain in hydrolysis. In practice a nucleoside residue in the polynucleotide is altered so as to activate the system for elimination. If the terminal residue is so altered, a method for stepwise degradation is available. If a nonterminal residue is suitably modified chain scission at that site can be effected. Normally in nucleic acid chemistry the activating function is a carbonyl group or derived function and this may be introduced into a nucleoside residue either by oxidation or by removal of the base.

1. *Stepwise Degradation of Polynucleotides*

A rational process for the stepwise degradation of a polyribonucleotide (**165**; R = remainder of chain) is based on periodate oxidation of the 3′-terminal residue to give the "dialdehyde" (**166**). Under mildly basic conditions this undergoes a β-elimination reaction to give **167** from which the terminal 3′-phosphate residue may be removed enzymically and the whole process then repeated [284–286]. This process has been extensively developed to increase the rate and the extent of reaction under milder conditions and to liberate the terminal base as such. The latter is achieved *inter alia*, by



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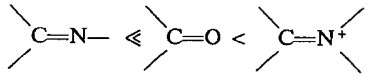
168 169 170

overoxidation with periodate in an amine-catalyzed reaction [287]. The perfection of the elimination reaction has come about by the introduction of primary amine catalysis at pH's around neutrality [288–292]. The amine, it appears [293,294], performs two functions, since high concentrations are efficacious; (1) the formation of an intermediate, and (2) acid–base catalysis. The reaction may be envisaged as proceeding through **168**, **169**, and **170** or closely related structures with the latter undergoing the elimination. Evidence for a cyclic intermediate is available, the stability of the latter being dependent on the nature of R′ (in $R'NH_2$). With methylamine the intermediate (**168**) is stable at pH 8–9 showing no sign of elimination, as would be expected [290,293]. Lowering of the pH to 5–7 leads to fast and essentially quantitative elimination in model experiments using adenosine-5′ phosphate. On the other hand reduction of the "dialdehyde" (**166**) with borohydride gives a stable system and, if $[^3H]BH_4^-$ is used, a labeled 5′-terminus. Reduction of an amine adduct gives a stable morpholine system which likewise cannot undergo elimination [293]. In this way an RNA can be derivated at its 5′-end, allowing for attachment to other molecules. Using this principle a ferritin–tRNA adjunct has been made [294a], and a nucleic acid–protein crosslink has been generated in 30 S ribosomes [294b].

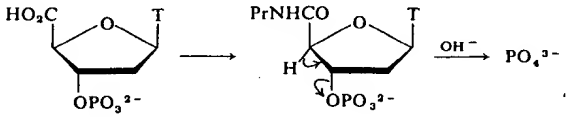
The point to note is that, so far as eliminations in general are concerned, the order of effectiveness for activation of the α-hydrogen by the carbonyl or derived function is



With very careful attention to detail [291] it has been possible to analyze several 3′-terminal RNA sequences, e.g., of TMV–RNA using aniline [295, 296], of phage f2 using cyclohexylamine [297–299], and of *E. coli* $tRNA^{Phe}$ using lysine as base [300]. In the latter work 26 cycles were performed, the first 19 nucleotides being sequenced unequivocally.

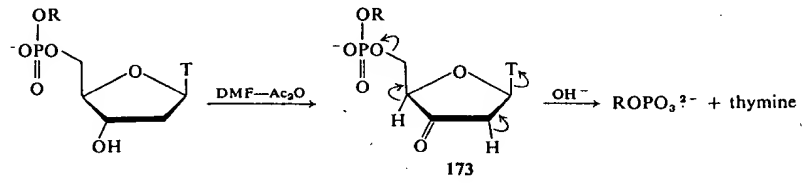
It is interesting that the terminal dialdehyde (**166**) and acyl hydrazides, such as thiosemicarbazide and isonicotinic hydrazide (isoniazid), react to give a product (**168**; R = NH·CO·C_5H_4N) essentially stable at neutrality, which may be used to label, and hence to identify the terminal sequence [301–303]. Dimedone reacts in the same way [304]. An extension of this is its use in attaching a large organic residue for various purposes such as purification of specific transfer RNA's [305]. Correspondingly functionalized cellulose columns may be used for isolation of periodate-oxidized nucleic acids or terminal oligonucleotides [306,307]. It may be that the efficiency of these latter processes could be increased. With careful pH control, a periodate-oxidized oligonucleotide may be attached in stable linkage to an aminoethyl-cellulose support and then be released (by β-elimination at lower pH) [298]. This is the basis of an automatic sequenator.

Stepwise degradation of polydeoxyribonucleotides has not yet been developed to a practically useful state. Oxidation of thymidine 3′-phosphate by platinum–hydrogen peroxide or –oxygen gives rise to the carboxylic acid (**171**). This residue (as the anion) is of course incapable of promoting a base-catalyzed elimination, although a decarboxylative elimination in this and the correspondingly oxidized dithymidine phosphate can be effected [308]. Derivatization (in two stages) to the propylamide (**172**) gives a weakly activated system. Quantitative elimination can be achieved under rather vigorous alkaline conditions [309].



171 **172**

Oxidation of the 3′-hydroxyl by dimethyl sulfoxide–acetic anhydride to the ketone (**173**) followed by treatment with alkali leads to the elimination of a



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5′-phosphoryl residue as well as the base residue, but the conditions, though milder than those used above, are still not satisfactory with polynucleotides [310]. Amine catalysis was apparently not investigated.

2. *Glycosidic Bond Hydrolysis*

So far as methods developed up to the present are concerned, chain scission has depended on the initial removal of a base residue. This is therefore a convenient place to discuss the hydrolysis of the glycosidic linkage.

As is well known, ribonucleosides are more stable than deoxyribonucleosides and, in each class, the purine bases are lost much more rapidly than the pyrimidines. Recently rate measurements made over a wide pH range have deepened our understanding of the process [311–317]. Figure 1 shows the pH-rate profiles for the hydrolysis of the natural deoxynucleosides. Individually, they show interesting variations in behavior. Thus thymidine shows both spontaneous and acid-catalyzed modes shared by deoxyuridine and the 5-bromo and iodo analogs [313,315,317]. The former is not observed with

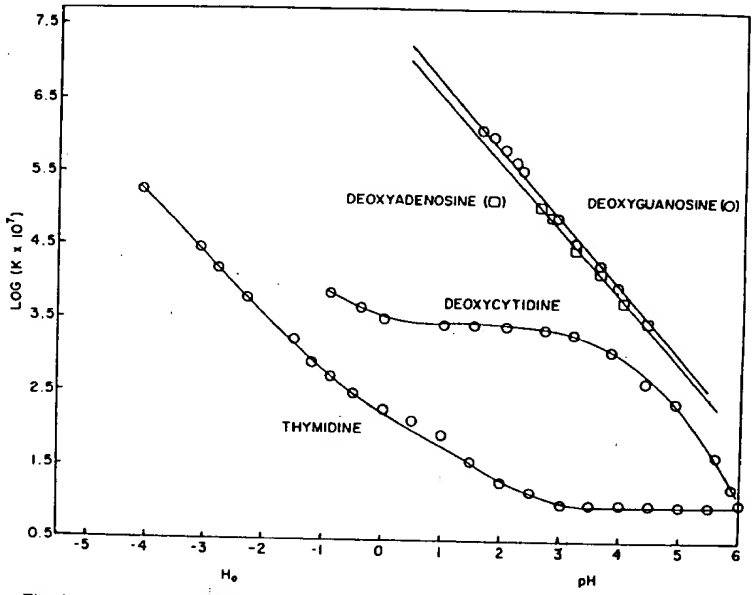
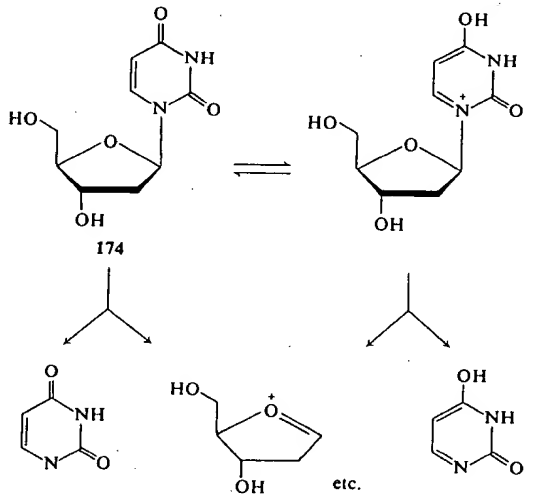


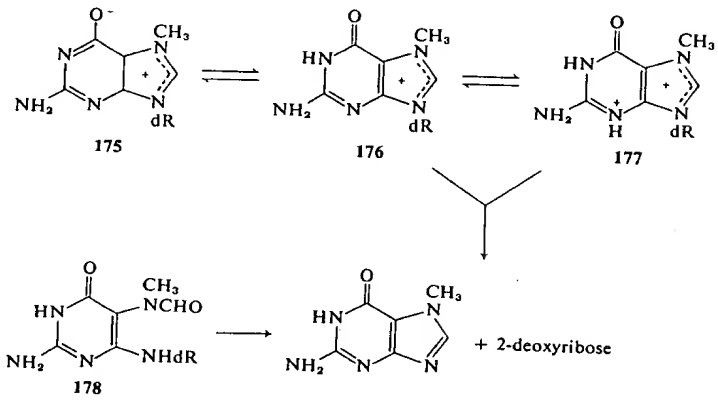
Fig. 1. Plot of the logarithms of the rate constants (sec^{-1}) at 95°C *vs* pH (above 0) and H_0 (below 0) for the hydrolysis of the four common naturally occurring deoxyribonucleosides [317].

deoxycytidine [317] but hydrolysis of the monocation (plateau at pH 0–3) and the dication occurs, as it does with deoxyguanosine and deoxyadenosine [316]. The two latter compounds have closely similar reactivities to acid hydrolysis and are more than 500 times more rapidly hydrolyzed than guanosine [316].

The observations fall into place if it is assumed that a preequilibrium protonation of the heterocyclic ring is followed by a rate-limiting ionization of the glycosidic bond, the deoxyuridine derivatives showing an additional water hydrolysis of the neutral species. Mechanistically hydrolysis of deoxuridine (**174**) is visualized as follows:



The effect of substituents at C-5 is in accord with this, i.e., electron-withdrawing halogens accelerate hydrolysis [311,315,317]. In the case of the purine derivatives preequilibrium protonation at C-7, the most basic site, is followed by a unimolecular cleavage of the N-9 to C-1′ bond [316]. Much evidence has been adduced in favor of this view [317,318] rather than the earlier one [319] that protonation of the sugar ring-oxygen is followed by opening of the lactol ring and generation of a Schiff's base which then undergoes hydrolysis. (For references to glycosylamine chemistry, see Capon [318].) Decisive evidence comes from a study of 7-methyldeoxyguanosine (**175**) and of 1,7-dimethyguanosine [316]. The former shows a pH-independent plateau in the pH-rate profile corresponding to the species (**176**). The latter is more than 25,000 times as reactive as guanosine. 7-Alkylation



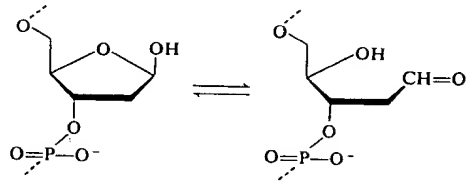
also leads to lability in the alkaline region [320,321]. Here reversible ring-opening of the imidazolium ring occurs. Though the glycosylamine bond in **178** is stable to base it is labile to acid, hydrolysis to 7-methylguanine competing with ring closure to **175**.

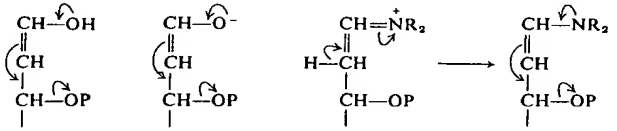
In addition to the above, 3-methyldeoxyadenosine has been shown to be more labile than 7-methyldeoxyguanosine in mild acid [120]. Deoxy-inosine and -xanthosine are said to be more labile than the corresponding adenine and guanine nucleosides from which they are derived by deamination.

In considering the stability of DNA, before and after alkylation and deamination, the new data on nucleoside hydrolysis are clearly very important.

3. *Eliminative Polynucleotide Chain Scission*

As mentioned above, removal of a base residue may be the first step in polynucleotide chain cleavage. Elimination is then activated by the unmasked sugar aldehyde group (**179**) or a derived function, and proceeds under acidic (**180**), basic (**181**), including amine (**182**) catalysis.





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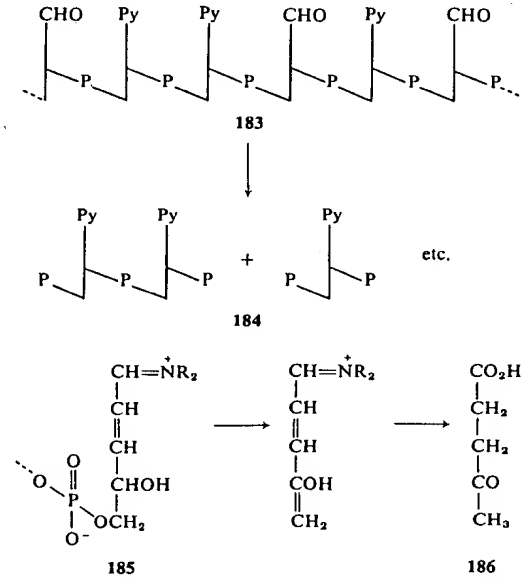
In this section we will first consider work with DNA. A variety of methods are available for removing base residues. In practice the purine residues are readily removed from DNA by mild acid treatment, for example, by HCl or formic acid [322,323]. The product, apurinic acid (**183**), which itself has suffered chain scission, may then be degraded further by acid [324] or, more cleanly, by an aromatic amine in aqueous formic acid. With the latter, purine removal and degradation of the formed apurinic acid is conveniently carried out in one step [325,326]. The products are of the form Py_nP_{n+1} (**184**). Using diphenylamine in aqueous formic acid at room temperature the products are



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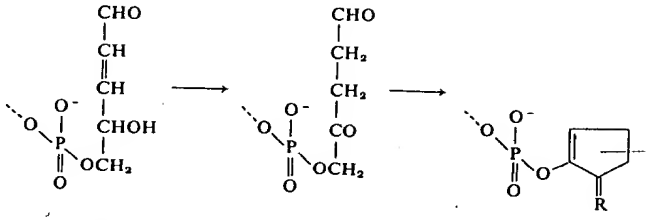
formed with no detectable contamination from products (Py_nP_n) resulting from the loss of the monoesterified phosphate or from the (slow) loss of pyrimidine residues. It is particularly interesting that the method leads to complete elimination of the free sugar residue, i.e., both 3′- and 5′-phosphoryl

groups are eliminated; phosphatase treatment in consequence gives products solely of the form Py_nP_{n-1}. Presumably the first intermediate (**185**) can undergo a second elimination efficiently from the vinylogous β-position, which in the acid-catalyzed reaction finally yields levulinic acid (**186**). The products of the diphenylamine/formic acid reaction can be separated into fractions of equal chain length (isostichs or isopliths) [327,328], DNA from φX174 gave pyrimidine sequences up to 11 nucleotides long [327], that from f1 up to 19 nucleotides long [329], and in the case of fd DNA a unique stretch of 20 nucleotides was formed and sequenced [330].

Degradation of apurinic acid with alkali gives chain cleavage but is less satisfactory since, as is well known, aldehydes, including free sugars, undergo a variety of reactions in base. In the reaction with apurinic acid some of the products apparently terminate in the residue (**189**; R = O) [331]. This result can be rationalized in terms of C_3—OP elimination to **187** followed by base-catalyzed prototropic shifts to give **188**, whence cyclization gives the cyclopentenone. Products terminating in (**189**; R = O) which is an enol phosphate, should be readily converted to the true elimination product by mild acid, though this has not been shown.



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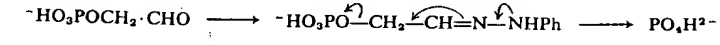
In attempts to bring about chain cleavage in apurinic acid under even milder conditions amine catalysis has been studied. Alphatic amines were essentially ineffective and hydroxylamine gave the stable oxime. Phenylhydrazine and semicarbazide were apparently effective but again the products were pyrimidine polynucleotides terminated by an organic residue, probably **189** (R = N·NHPh or N·NH·CO·NH_2) [332,333]. It is not quite clear why phenyl hydrazine should lead to elimination; it and *unsym* dimethylhydrazine are explicably excellent catalysts for α-elimination, e.g., from glycolaldehyde phosphate [334,335], viz:



When DNA is treated with hydrazine the pyrimidine bases are degraded (see Section II, B), and the sugar residue remains almost certainly as the

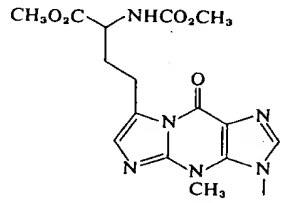
hydrazine derivative [336,337]. There are conflicting reports as to the stability of this system to base [337,338]. Acid-catalyzed degradation is precluded by the lability of the purine glycosidic bonds. However, pretreatment with benzaldehyde to displace the hydrazine [339], then base, certainly allows chain cleavage to occur. The whole process has been subjected to careful scrutiny and it is evident that the reaction does not go entirely quantitatively [340]. Purine polynucleotides (Pu_nP_{n+1}) are formed and can be separated into their isopliths [341]. There is a relatively good correlation between the polypurine and polypyrimidine isopliths as there should be if both are obtained by degradation of the same complementary double-stranded DNA [337,341]. The disadvantages of strong base-catalyzed elimination are obvious. More recently it has been claimed that aromatic amines, aniline, or *p*-anisidine, under mild (pH 5) conditions give chain rupture. With apyrimidinic acid, isopliths ($Pu_nP_{n=1}$) of n = 1–8 can be recognized but these too, it seems, may carry a terminal non-nucleotidic organic residue [342]. This may, in certain applications, be no disadvantage since, for instance, it may allow radioactive labeling with, e.g., ^{14}C-aniline.

Any chemical reaction which degrades a base residue is, in principle, capable of application to DNA chain cleavage. Those above have been studied most extensively. Other methods, particularly those involving base oxidation, have been given some consideration. Osmium tetroxide oxidizes thymine residues much faster than the other bases [9,343]. As applied to DNA, the oxidized residue can be removed by base, whence diphenylamine–formic acid treatment affords groups of cytosine nucleotides, C_nP_{n+1} [344]. Sequences of adenine nucleotides, as yet not sufficiently characterized result from permanganate oxidation, followed by alkali [345]. The problem associated with these reagents is that, as in hydrazinolysis, a nitrogenous residue may be left attached glycosidically and this has to be removed completely before the chain cleavage reaction can be initiated. As yet, it would seem, solutions to this problem are only partial.

Turning to RNA we find that much less work has been devoted to the question of chain cleavage. This is not surprising as ribonucleases of high specificity abound so that the need to devise chemical cleavage reactions is less pressing. The same kind of approach as has been taken with DNA is possible, provided always that it is recognized that in RNA internucleotide linkages are much less stable and the glycosidic linkages are much more stable than those in DNA. This places considerable restrictions on the methods available. The base and acid lability of the internucleotide linkage precludes the preparation of riboapurinic and riboapyrimidinic acid. Removal of uracil from RNA by hydroxylaminolysis at pH 10 [59,346] gives a product which, after mild acid treatment (to remove the oximino residue), can then be cleaved by aromatic amine-catalyzed elimination [347]. Again a terminal

non-nucleotide residue is retained, and the hydroxylaminolysis step leaves much to be desired [348].

Probably the best example of chain cleavage as applied to RNA depends upon the very high degree of acid lability of the base Y (**190**) in yeast $tRNA^{Phe}$ [349]. The tRNA contains 76 bases and base Y which is in position 37 is



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removed by 0.1 *N* HCl at room temperature [350]. The aniline-catalyzed elimination then cleaves the RNA chain with, apparently, no other damage to the system. Indeed also present in this tRNA is a 7-methylguanine residue at position 50. Somewhat stronger acid treatment removes it as well and elimination of the intervening oligonucleotide can be effected [351].

4. *Thermal Degradation of Polynucleotides*

When a biologically active nucleic acid is heated in solution (or sometimes in the dry state) the rate of inactivation is greater at higher temperatures and lower pH values. The process is irreversible and is to be distinguished from denaturation at the melting temperature in double-stranded DNA. Many studies of subcritical heating have been made [352]. The process in DNA is considered to depend on loss of purine bases, guanine rather faster than adenine, and as a consequence, chain scission. Both can be measured [352–355]. In circular single-stranded ϕX174 DNA the rate of depurination and of inactivation are similar and are considered to be distinguishable from the subsequent scission step [356,357]. Poly dAT undergoes slow chain scission (~pH 4) which is associated with loss of adenine and appearance of terminal 3′-phosphate residues [358]. A considerable increase in rate is observed at temperatures between 60° and 70°C, i.e., around the T_m value, so that secondary structure protects against depurination. The pH-rate profiles for the acid-catalyzed hydrolysis of purine deoxyribonucleosides (Section IV,D,2) are consistent with slow glycosidic bond hydrolysis at, or somewhat below, neutrality. Both this step and the subsequent elimination reaction leading to chain scission may involve general species catalysis.

Alkylation at N-7 in guanine and N-3 in adenine residues, as we noted, leads to much-enhanced rates of hydrolysis and in DNA a slow release of

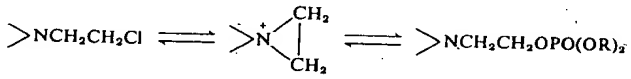
alkylated purines occurs [120,359,360]. Inactivation results from alkylation. Indeed following alkylation by EMS, bacteriophage T4 survival studies show a primary and a delayed inactivation which are reasonably ascribed, respectively, to the alkylation and the subsequent chain cleavage [361]. Alkylation mutagenesis presents a confused picture [188,362,363]. In addition to the question of the site of alkylation, explanations require consideration, *inter alia*, of replication and of repair error before and after depurination. Since both transitions and transversions occur it is probable that more than one mechanism is involved [188].

In contrast to DNA, thermal degradation of RNA appears to proceed by direct hydrolysis of internucleotide bonds [254]. The pH-inactivation rate profile for TMV–RNA differs markedly from that for ϕX174 DNA [357] and evidence for the generation of terminal 3′- or 2′,3′-phosphate end groups has been given [364].

E. Alkylation on Phosphate

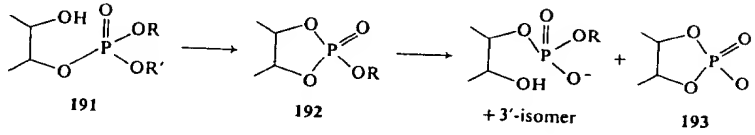
We have seen that removal of a base from a nucleic acid leaves a sugar residue which is then a point of lability in the system. Alkylation of guanine at N-7 or of adenine at N-3, as occurs in DNA, can lead to this result. Alkylation of the internucleotidic phosphoryl group can, in principle, also lead to chain cleavage, but for a different reason. In the latter lability is introduced because of the much greater ease of hydrolysis of phosphate triesters than of diesters.

The stability of an alkylated internucleotide linkag will depend very much on whether we are dealing with the deoxyribo- or the ribo- series. In the former, the system will be a normal trialkyl phosphate and thus will be susceptible to neutral or base-catalyzed hydrolysis. This may be quite slow (for some rates, see Cox and Ramsay [365]) and it is likely that the introduced alkyl group will often be the more easily displaced. The ease of base-catalyzed displacement, broadly, is in the order 1° > 2° > 3°; more accurately it reflects the stability of the alkoxide ion formed or, in other words, the acidity of the corresponding alcohol. Thus of the simple alkoxy groups, methoxy is the most easily displaced. It follows that chain cleavage is not a necessary consequence of *P*-alkylation since the newly introduced group may be more easily lost in the triester. It is interesting that the neutral hydrolysis of trimethyl phosphate proceeds by alkyl–oxygen cleavage, in effect, acting as an alkylating agent. This is particularly the case in alkylation by *N*- and *S*-mustards and imines, since the reaction is reversible, 2-aminoethyl



dialkyl phosphates, themselves being alkylating agents [88,366]. *P*-Alkylation by a Michael reaction, for instance by acrylonitrile, should in principle occur reversibly, though the question does not seem to have been studied in connection with polynucleotides. Information is rapidly accumulating on the stability of internucleotide triesters as a result of increased interest in intermediates carrying such groupings in the chemical synthesis of oligonucleotides (see Chapter by Letsinger in Volume III).

Alkylation of the internucleotide linkage in a polyribonucleotide must give an exceedingly labile system. Model experiments show that a nucleoside 2′ (or 3′)-dialkylphosphate (**191**) is unstable over the entire pH range [367]. The internucleotide linkage could be broken at two stages; (1) during the formation of the cyclic intermediate (**192**) and (2) during the hydrolysis of the



latter. Opening of 5-membered cyclic triesters is exceptionally fast and concomitant hydrolytic cleavage of the exocyclic P—O bond to give **193** is also largely accelerated [245].

Having discussed the expected results of forming phosphotriester groups in polynucleotides, we should now look at the evidence as to whether it does or does not occur. It is evident from model experiments that alkylating proceeds very much more readily on base residues than on phosphate residues. The nucleophilicities of dialkyl phosphate anions (a model for the internucleotide linkage) are relatively low. Table III gives a basis for comparison with other nucleophiles [368]. Monoalkyl phosphates (models for terminal residues) are more reactive. Their reactivity is pH-dependent, the dianion, naturally, being more nucleophilic than the (protonated) monoanion.

TABLE III

Competition Factors of Some Phosphates for *N,N*-Di-2′-chloroethyl-2-naphthylamine[a]

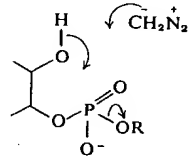
HPO_4^{2-}	108	$HOCH_2CH(OH)CH_2OPO_3^{2-}$	66
$H_2P_2O_7^{2-}$	45	$(C_2H_5O)_2PO_2^-$	5.3
$C_2H_5OPO_3^{2-}$	153	$[HOCH(CH_3)CH_2O]_2PO_2^-$	3.0
$HOCH_2CH_2OPO_3^{2-}$	100	CH_3COO^-	100
$HOCH(CH_3)CH_2OPO_3^{2-}$	134		

[a] From Davis and Ross [368].

These considerations apply to Me_2SO_4, alkyl sulfonates and the like. On the other hand, diazoalkanes react, in effect, with the free acid. Since the secondary phosphoryl dissociation of phosphomonoesters is around 6, at neutrality there will be about 10% of the monoprotonated form present. Adenosine-5′ phosphate at pH 7 gives essentially only the monomethyl ester with diazomethane, while with Me_2SO_4 (especially at pH 5) N_1-methylation is very much favored. With cytidylic acid *P*-alkylation alone occurs with diazomethane; with Me_2SO_4 alkylation is mainly at N-3 [108].

Work with dinucleoside phosphates, both in the ribo- and the deoxyribo-series make it clear that methylation by both Me_2SO_4 and diazomethane occurs selectively on the base residue and that there is no *P*-alkylation. There is in consequence, no internucleotide bond scission. This view has been contested in regard to diribonucleoside phosphate alkylation but a careful reexamination has confirmed the original findings [369]. Thus at least from the point of view of preparative chemistry the internucleotide linkage in solution at neutral pH is extremely unreactive; ApU for instance gives ApMeU quantitatively with diazomethane and MeApU with dimethyl sulfate. Homopolynucleotides are also readily alkylated. In no case has any direct evidence of internucleotide *P*-alkylation been found and so the process is preparatively useful. The terminal 5′-phosphate in poly U is alkylated by sulfur mustard [370] and, one may presume, by other alkylating agents. Some workers have carried out alkylations with a variety of reagents without, however, commenting on any changes in sedimentation constant [111,371, 372]. Others have noted that diazomethane methylation of poly U but not poly A [108] and of rRNA and tRNA leads to some degradation [373]. Ethyl methane sulfonate ethylation of poly A gives rise to some chain scission [114].

In the case of diazomethane, chain scission may be dependent on *P*-alkylation; on the other hand, the interesting suggestion has been made that the diazomethane is acting as a base as in **194** [108].



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Chain scission of DNA as a result of alkylation certainly occurs. Whether this is due to *P*-alkylation in addition to events following base modifications is unclear [374]. But it should be reiterated that, on chemical grounds alkylation on the internucleotide linkages of ribo- and deoxyribopolynucleotides must occur; the nucleophilicity of dialkyl phosphates is low but finite. It was a matter only whether a sensitive enough direct test could be devised. It has recently been shown that at a level of alkylation of DNA by EMS and MMS, introducing 26–28 groups/10^6 daltons, 10–15% of the alkyl groups are accounted for as alkyl phosphate after acid hydrolysis. The corresponding alcohol can be released from the latter by phosphatase treatment [374a].

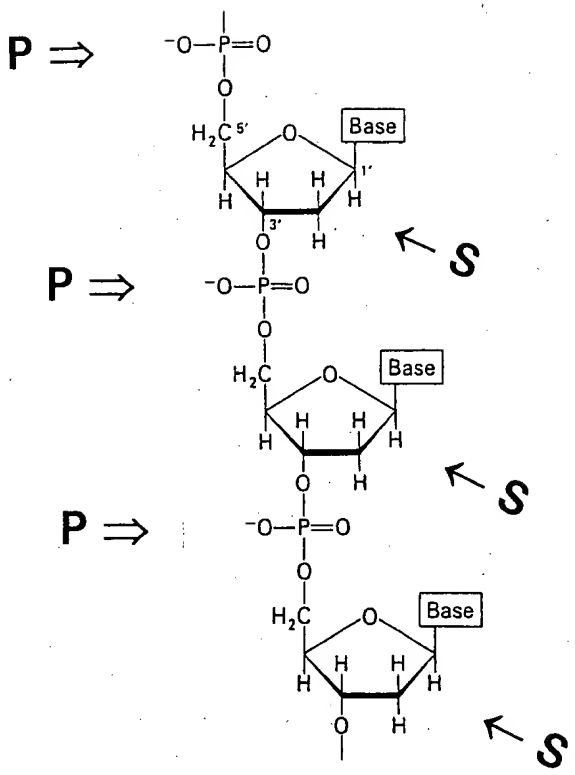
Alkylation on the terminal mono, pyro, or triphosphate group of polynucleotides *in vivo* is also a very probable event and has not been sufficiently considered as a cytotoxic process.

V. Influence of Conformation on Reactivity in Polynucleotides

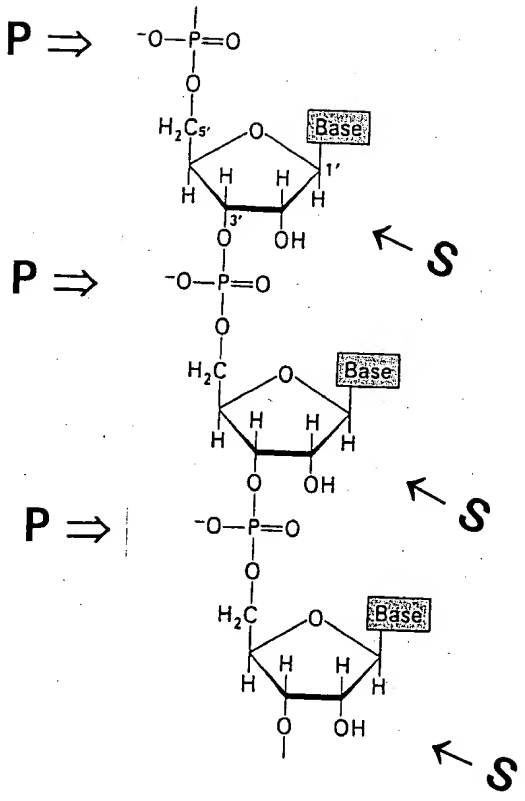
A. Methods of Study

The extent to which a polynucleotide is modified in a reaction will largely determine the way in which the process is studied. At one extreme, a mutagenic response provides strong evidence for reaction. Further genetic analysis of a mutant may allow classification into one or other of the several types of lesion and this, in turn, with the help of knowledge of the chemical specificity of the reagent, may lead to conclusions as to the nature of the primary event. Such conclusions must inevitably be tentative since the reaction, per se, cannot at present be studied in a direct way. Likewise changes in the hydrodynamic, or other physical behavior of nucleic acid solutions are very sensitive to structural alterations. Such methods give evidence of chain cleavage, but the nature of the alteration is necessarily obscure. For example, unwinding of supercoiled DNA as a result of one single-strand break is readily observed. Bisulfite-Pb^{2+} causes such a strand cleavage [375]. A possible hypothesis that this is due to radical oxidation of a sugar residue, may be inferred from the known chemistry of bisulfite but this remains a conjecture. What matters is that incredible sensitivity is available in this and a variety of other potential test systems which involve chain breakage. Interstrand crosslinking at very low levels can be demonstrated by the resultant rapid reannealing [191,352].

At the next level, small extents of reaction of bases can be detected and quantitated by using either radioactively labeled reagent or nucleic acid. Labeled reagent is much used, for example, in alkylation studies and in this instance product ratios can be accurately measured. Using unlabeled reagent and ^{32}P-nucleic acid is convenient and more economical since the reagent is very often in large molar excess over the substrate and in general is not recovered. Quantitation is conveniently carried out at the mono- and oligonucleotide level [376].



Structure of part of a DNA chain.



Structure of part of an RNA chain.